Isolation and characterization of a novel population of potential kidney stem cells from postnatal mouse kidney

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

by

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Abstract

The limited capacity of the kidney for regeneration and the scarcity of organs for transplantation have led to the search for alternative therapies to repair the damaged kidney before it becomes non-functional. One approach to regenerate damaged structures in the kidney would be to use stem cells. However, the existence of stem cells in mature kidneys is still controversial.

During the first part of this study the prevalence and location of renal stem cells during mouse development was established using Pax-2 immunostaining. Pax-2 positive cells were found in the outer cortex and the papilla of neonatal mice. By week one Pax-2 positive cells were restricted to the papilla. This Pax-2 expressing population, which remained in kidney after nephrogenesis was complete, may be similar to the precursor cells previously found in human kidney.

Pax-2 is expressed in both the nephrogenic metanephric mesenchyme (MM) and the non-nephrogenic ureteric bud, however, the marker WT1 is only expressed in MM. To determine the origin of Pax-2 positive cells, WT1-Cre/Rosa26R mice were used, in which cells expressing WT-1 also express Cre recombinase which irreversibly activates β-galactosidase. Day 7 mice were immunostained for Pax-2 and β-galactosidase. When transgenic mice were analyzed, some Pax-2 positive cells in the papilla were found positive for β-galactosidase, suggesting that they were derived from metanephric mesenchyme.

Magnetic cell sorting was the chosen technique to select Pax-2 positive cells in kidney, however, a cell surface marker was required to do so. A screening of twenty-one lectins showed that peanut agglutinin lectin (PNA) bound to the majority of Pax-2 positive in the papilla. PNA was then used for the isolation of the cell population of interest by immunomagnetic cell sorting.

However, expansion of disaggregated cells from mouse kidney under the optimal culture conditions proved to be equally effective to enrich the population of Pax-2 positive cells or putative kidney stem cells (pKSC).

These expanded populations displayed some potential to integrate into new developing kidneys. It was possible to induce pKSC to differentiate into more specialized cell types, such as tubular epithelial cells, podocytes or mesangial cells, by changing the culture conditions. These cells also have high clonogenic potential, which is characteristic of stem cells.

Glossary of common abbreviations

α-SMA	Alpha smooth muscle actin
ANOVA	Analysis of variance
AP	Alkaline phosphatase
AQP1	Aquaporin-1
AQP2	Aquaporin-2
BMP-7	Bone morphogenic protein 7
BrdU	Bromodeoxyuridin
BSA	Bovine serum albumin
CD2AP	CD2-asssociated protein
DAPI	4′,6-diamidino-2-phenylindole, dihydrochloride
DMEM	Dulbecco's modified Eagle's medium
DNase I	Deoxyribonuclease I
dpKSC	Differentiated putative stem cells
dSTO	Sodium deoxycholate-treated STO
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ESRF	End stage renal failure
FACS	Fluorescence-activated cell sorting

FCS	Fetal calf serum
FGF	Fibroblast growth factor
FN	Fibronectin
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBM	Glomerular basement membrane
GDNF	Glial cell-line derived neurotrophic factor
HBSS	Hanks' buffered saline solution
HGF	Hepatocyte growth factor
KSC	Kidney stem cells
Ksp-cadherin	Kidney specific cadherin
LIF	Leukemia inhibitory factor
LRC	Low rate cycling cells
MACS	Magnetic-activated cell sorting
MEF	Mouse embryo fibroblasts
MEM	Modified Eagle's medium
MM	Metanephric mesenchyme
NCAM	Neural cell adhesion molecule
NT	No template control
PBS	Phosphate buffered saline
PCNA	Proliferative cell nuclear antigen
PECAM	Platelet endothelial cell adhesion molecule

PFA	Paraformaldehyde
pKSC	Putative kidney stem cells
Sall1	Sal like 1 gene
siRNA	Small interfering RNA
SP	Side population
STO	Sandoz inbred mouse embryo-derived thioguanine and ouabain- resistant cells
STO-CM	STO-conditioned media
TAE	Tris-acetate-EDTA
TBS	Tris buffered saline
TGFα	Transforming growth factor alpha
TGFβ	Transforming growth factor beta
THP	Tamm-Horsfall protein
TIMP2	Tissue inhibitor of metalloproteinase 2
UB	Ureteric bud
VEGF	Vascular endothelial growth factor
vWF	von Willebrand factor
WT1	Wilms' tumor suppressor gene
ZO-1	Zonula occludens-1

Chapter 1: Introduction

Some organs in mammals have the ability to regenerate to compensate for damage; for example the liver is able to regenerate when a fraction is removed (Stanger *et al.*, 2007). Other organs, such as skin or blood, also present an evident capacity for regeneration (Clark and Kamen, 1987; Li *et al.*, 2004). However, most mature organs, such as pancreas, heart or kidney, have a very limited regenerative potential (Stanger *et al.*, 2007). When extensive insult occurs in these organs, the damage is usually irreversible and leads to failure of the organ, in such cases transplantation is the chosen therapy (Schaubel *et al.*, 2000; Schnuelle *et al.*, 1998). Thousands of people die every year in Europe waiting for organ transplants (Ransford *et al.*, 2000). The lack of available organs for transplant necessitates the search for alternative treatments.

During the last decade it has been suggested that repair or regeneration of damaged organs with stem cells might offer a suitable alternative to transplantation. There have been several successful attempts during the last few years in regenerative medicine. In 2006, Anthony Atala's group from Wake Forest University (North Carolina) grew a whole bladder *de novo* using urothelial and smooth muscle cells isolated from the patient, and then successfully transplanted the bladder into the patient (Atala *et al.*, 2006). Another exciting breakthrough was achieved when Macchiarini *et al.* (2008) in Spain managed to reconstruct and replace a bronchus using bone marrow stem cells from the patient.

The kidney is one of the organs that have a limited regeneration potential in mammals (Drummond, 2003). When the kidney is injured and loss of renal function occurs, the outcome may be endstage renal failure (ESRF) (Schnuelle *et al.*, 1998). While dialysis provides a means of replacing the loss of kidney function for patients in ESRF the only current means of permanently treating ESRF is transplant (Schnuelle *et al.*, 1998).

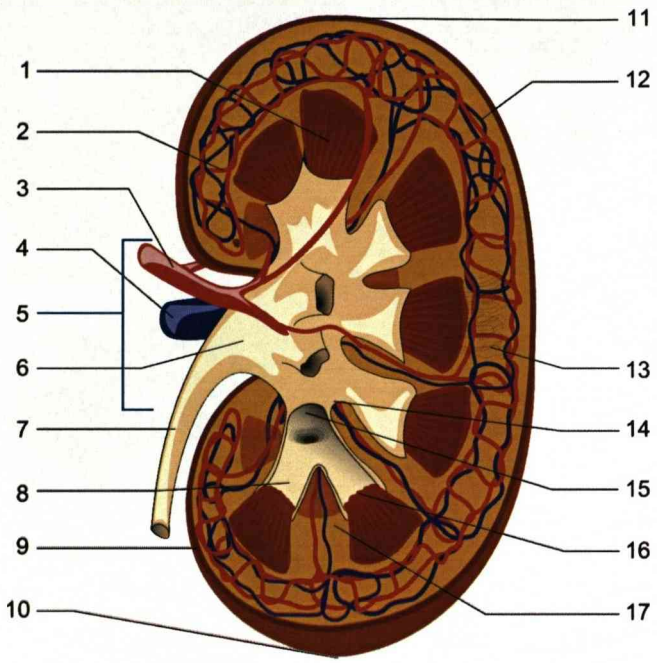
There is severe lack of kidneys available for transplantation requiring many patients be treated with dialysis. Furthermore, dialysis requires expensive machinery, severely reduces the patient's quality of life, and, most importantly, has significant side effects for the patient including osteoporosis (Barreto *et al.*, 2006). These considerations have led to the search for potential regenerative treatments.

One proposal is that it might be feasible to repair or replace damaged nephrons in patients suffering chronic renal failure before the kidney becomes non-functional using adult stem cells from the patient. This approach would have the advantage that no rejection would occur when transplanted. However, the existence of stem cells in mature kidneys is still controversial.

The kidney is a highly complex organ and in order to develop a therapy using stem cells to repair or regenerate injured kidney, it is very important to understand both the anatomy of the fully mature kidney and how the kidney develops.

1.1 Anatomy of the Kidney

The kidneys are bean-shaped organs located in the retroperitoneal space (Figure 1-1) and are covered by a layer of connective tissue called the capsule, the main components of which are fibroblasts, myofibroblasts and collagen fibers (Martini and Bartholomew, 1997).



1. Renal pyramid
2. Interlobar artery
3. Renal artery
4. Renal vein
5. Renal hylum
6. Renal pelvis
7. Ureter
8. Minor calyx
9. Renal capsule
10. Inferior renal capsule
11. Superior renal capsule
12. Interlobar vein
13. Nephron
14. Minor calyx
15. Major calyx
16. Renal papilla
17. Renal column

Figure 1-1 Diagram showing gross structures of a human kidney (From Piotr Michał Jaworski with permission under the terms of the GNU Free Documentation License, http://commons.wikimedia.org/wiki/File:Kidney_PioM.png)

The nephron is the basic structural and functional unit of the kidney and is formed by the renal corpuscle (Bowman's capsule and glomerulus) and the renal tubule (Figure 1-2) (Georgas *et al.*, 2008; Vize *et al.*, 2003). The renal corpuscle is responsible for the filtration of blood, which generates an ultrafiltrate (Junqueira *et al.*, 1977). The tubular portions are then responsible for altering the concentrations of ions and other chemicals in the ultrafiltrate to produce dilute

urine. This process is essential not only for waste excretion but also for maintaining the appropriate ion balance in the body.

The Bowman's capsule is the initial portion of the nephron and it is invaded by the vascular tuft, which is the glomerulus. The renal corpuscle contains the filtration apparatus of the kidney, which comprises three components: a vascular endothelial cell layer, an outer epithelial cell layer that contains specialised cells

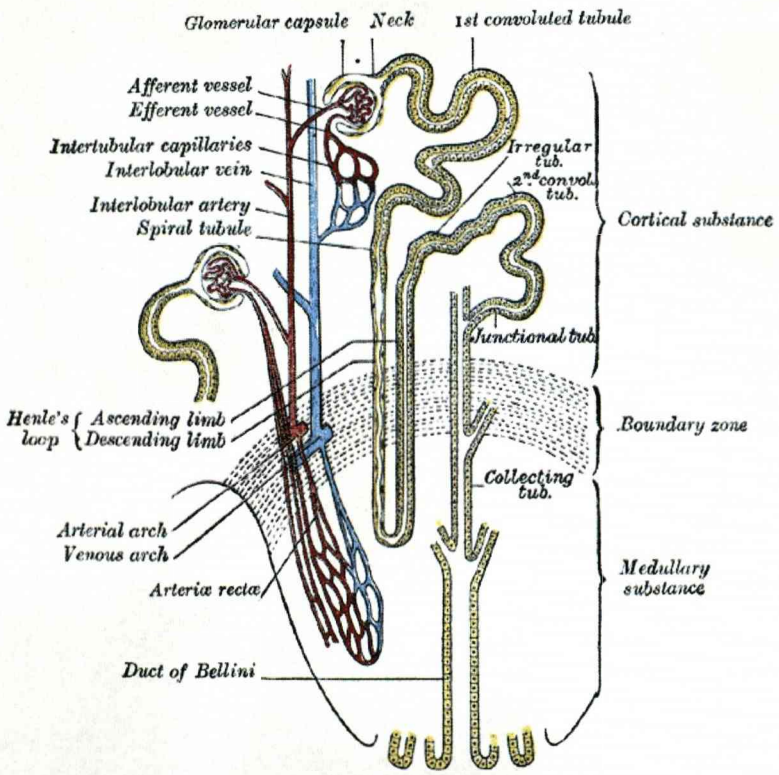


Figure 1-2 Diagram showing the structures of the nephron (Gray, 1918). This image is in the public domain because its copyright has expired obtained from http://commons.wikimedia.org/wiki/File:Gray1128.png reproduced from 20th U.S. edition of Grays Anatomy of the Human Body (1918).

called podocytes, and an intermediate layer of basement membrane called glomerular basement membrane (GBM) (Vaughan and Quaggin, 2008).

The structural support that the GBM contributes to the glomerulus is responsible for its characteristic spherical morphology (Gundersen and Osterby, 1980). Glomerular endothelial cells and mesangial cells are located inside the GBM, while podocyte cells lie outside(Smoyer and Mundel, 1998).

The kidney can be divided into three different regions that are clearly distinguishable by the naked eye, given the differences in blood supply within the organ. The outer part of the kidney is the cortex, which receives 90–95% of the blood passing through the kidney and therefore has a characteristic intense red colour (Regan *et al.*, 1995). The cortex contains the renal corpuscles with the convoluted and straight tubules of the nephron as well as collecting tubules and collecting ducts (Vize *et al.*, 2003).

The renal medulla forms the inner part of the kidney and can be divided into the outer and inner medulla, based on which portions of the renal tubules are more abundant (Liu *et al.*, 2001; Terada and Knepper, 1989). The outer medulla can also be divided into two: the outer and the inner stripe. The outer stripe of the outer medulla contains the straight part of the proximal and distal tubules, the thick ascending limb of the loop of Henle and the collecting ducts (Vize *et al.*, 2003). The inner stripe of the outer medulla contains the thin descending limbs of the loop of Henle, the collecting ducts and the thick ascending limbs. The inner medulla contains the thin descending and ascending limbs and large collecting ducts, which empty into the renal calyx (Vize *et al.*, 2003).

The tubules in the renal medulla are arranged in a morphology that resembles a cone and they are referred to as the renal pyramids. The apical portion of the

renal pyramids, known as the papilla, is where the collecting ducts open into the minor calyces (Martini and Bartholomew, 1997). The minor calyces then open into the major calyces, which are in turn subdivisions of the renal pelvis (Martini and Bartholomew, 1997; Vize *et al.*, 2003).

The juxtaglomerular apparatus can be found next to the vascular pole of the glomerulus and adjacent to the efferent and afferent arterioles. The juxtaglomerular apparatus is composed of extraglomerular mesangial cells, juxtaglomerular cells and the macula densa. The macula densa are specialised tubular cells from a fraction of the distal convoluted tubule (Martini and Bartholomew, 1997). The macula densa measures the ionic concentration of fluid in the distal convoluted tubule and controls the release of renin by the juxtaglomerular cells. This is a crucial component in the regulation of blood volume and pressure via the renin-angiotensin-aldosterone system.

1.1.1 Major cell types of the nephron and their markers

The nephron can be divided into the renal corpuscle, comprising the Bowman's capsule and glomerulus, and the different tubular fractions (Georgas *et al.*, 2008). Each portion of the nerphron is characterised by highly specialized epithelial cells with specific functions. The different functions of the cell types leads them to express specific proteins depending on their position on the nephron. (Vize *et al.*, 2003). These proteins can be used as markers to identify different cell types from the nephron (Vize *et al.*, 2003).

Podocyte cells

The epithelial podocytes lie on the outer layer of glomerular capillaries (Figure 1-3), and are derived from the metanephric mesenchyme (MM), one of two embryonic tissues responsible for kidney development (see Kidney Development page 30). Podocytes can be divided into three structural and functional segments: the cell body, major processes, and foot processes (Smoyer and Mundel, 1998). The cell bodies and major processes of podocytes float in the filtrate within the Bowman's capsule, whereas the foot processes are in direct contact with the GBM and interdigitate closely with foot processes from other podocytes (Smoyer and Mundel, 1998; Vize *et al.*, 2003). Filtration slits are present between the foot processes, which are bridged by the slit diaphragm (Smoyer and Mundel, 1998).

The slit diaphragm is a thin structure that connects the foot processes of the podocytes and plays a crucial role in glomerular filtration. The slit diaphragm is involved in size selectivity and is responsible for restricting protein passage through the glomerular filtration barrier (Miner, 2002; Reiser *et al.*, 2000).

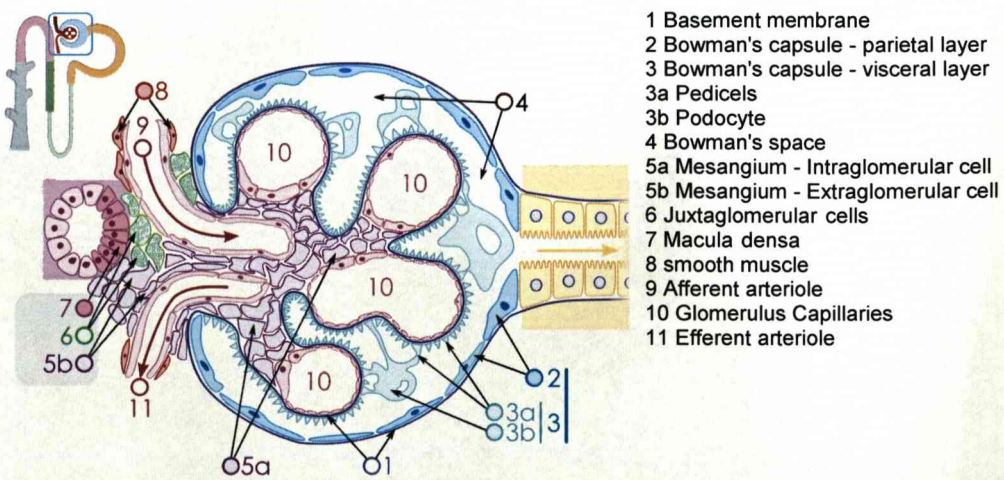


Figure 1-3 Diagram showing detailed histological structure of the glomerulus (from Michał Komorniczak with permission under the terms of the Creative Commons Attribution Sharealike 3.0 license, http://en.wikipedia.org/wiki/File:Renal_corpuscle.svg).

Podocytes are highly specialized cells that perform a variety of glomerular functions such as GBM turnover, maintenance of the glomerular filtration barrier, support of the capillary tuft, and regulation of glomerular filtration (Mundel and Kriz, 1995).

Above the slit diaphragm of the podocytes lies the glycocalyx, a layer of negatively charged glycoproteins that repulse negatively charged proteins in the glomerular capillary (Smoyer and Mundel, 1998). As a result of these repulsions, there is less filtration of these negatively charged proteins than uncharged proteins of the same size (Smoyer and Mundel, 1998). The glycocalyx is also essential for maintaining the structure of the foot processes (Smoyer and Mundel, 1998). The transmembrane sialoprotein podocalyxin is one of the major proteins of the glycocalyx, and helps to maintain structure and function of the podocyte foot processes (Vize *et al.*, 2003). Differentiating podocytes synthesize podocalyxin (Schnabel *et al.*, 1989) and its expression is restricted to the apical surface of foot processes (Schnabel *et al.*, 1989; Vize *et al.*, 2003).

During the early stages of nephron development the developing podocytes are present as a simple layer of cuboidal or columnar epithelium. As the nephrons mature, intercellular junctional complexes migrate basolaterally where they are eliminated or substituted by slit diaphragms which bridge the filtration slits between foot processes (Schnabel *et al.*, 1990; Vize *et al.*, 2003).

The tight junction protein, zonula occludens-1 (ZO-1), is expressed in all the portions of the renal tubules at the tight junctions in the junctional complexes, but is most abundant in glomeruli where it is concentrated at the point where the slit

diaphragms insert into the plasmalemma of the foot processes (Schnabel *et al.*, 1990). It seems that slit diaphragms are derived from tight junctions and their functional property is to define the boundary between plasmalemmal domains (Schnabel *et al.*, 1990).

Other proteins that are required for normal development of the foot processes include neph1, nephrin and CD2-associated protein (CD2AP). Neph1 is a slit diaphragm protein that forms a protein complex with nephrin, a transmembrane receptor that is present at the intercellular junctions of the foot process (Barletta *et al.*, 2003). Deletion of neph1 or nephrin results in abnormal development of foot process junction formation (Garg *et al.*, 2007; Georgas *et al.*, 2008). CD2AP is another protein of the slit diaphragm that is critical for glomerular integrity. Mice lacking this protein develop abnormal foot processes, and die of renal failure six to seven weeks after birth (Shih *et al.*, 1999).

Other proteins have also been proposed as differentiated podocyte markers such as WT1, vimentin, and synaptopodin (Shankland *et al.*, 2007; Vize *et al.*, 2003). The expression pattern of the transcription factor WT1 in glomerular podocytes during mouse adulthood indicates a possible role in maintaining functional podocytes (Mundlos *et al.*, 1993). Point mutations in the WT1 gene in humans are associated with impaired podocyte function leading to damage in the glomerulus (Mundlos *et al.*, 1993). WT1 expression is also crucial in controlling various stages of nephrogensis as described below in Kidney Development (page 30).

The intermediate filament protein, vimentin, is highly expressed in podocytes and vasculature. Vimentin does not appear to have an essential role in the kidney under normal physiological conditions, but following induced renal injury in mice, vimentin-null mice rapidly developed ESRF whereas wild-type mice recovered (Terzi *et al.*, 1997). During kidney development vimentin expression is first seen in undifferentiated metanephric mesenchyme. As nephrogenesis progresses, vimentin is only expressed in the differentiating podocytes of the nephrons. In mature nephrons, vimentin expression is restricted to podocytes; however, it is only observed in cell bodies and major processes and it is absent in foot processes (Oosterwijk *et al.*, 1990; Vize *et al.*, 2003).

Synaptopodin is an actin associated protein that is specifically expressed in post-mitotic differentiated podocytes and absent in immature podocytes and progenitors (Mundel *et al.*, 1997a; Shankland *et al.*, 2007). Synaptopodin is present in the foot processes of podocytes where its association with actin microfilaments is involved in the formation of the characteristic long unbranched actin filaments that can be observed in differentiated podocytes (Asanuma *et al.*, 2005).

The capacity of podocytes to repair or regenerate after injury is very limited (Mathieson, 2003). Under normal circumstances podocytes do not have the ability to proliferate and repopulate denuded areas (Barisoni and Mundel, 2003). The inability to repair podocyte injury means that damage to the glomerulus eventually leads to loss of glomerular function resulting in progressive glomerular disease (Barisoni and Mundel, 2003).

Mesangial cells

Mesangial cells are glomerular intercapillary cells that occupy a central position in the glomerulus (Figure 1-3) (Schlondorff, 1987; Vize *et al.*, 2003). Mesangial cells, like podocytes, are derived from MM. Mesangial cells have characteristics of modified smooth muscle cells, and are therefore able to contract and relax in response to vasoactive agents in order to modify glomerular filtration locally (Schlondorff, 1987). In addition to expressing α-smooth muscle actin (αSMA) (Skalli *et al.*, 1989; Stephenson *et al.*, 1998), mesangial cells also express the intermediate filament protein desmin (Georgas *et al.*, 2008). Desmin is highly expressed in skeletal and cardiac muscle, therefore its presence in mesangial cells is probably related to the contractile function of these cells (Georgas *et al.*, 2008).

Mesangial cells play various important roles in the kidney. As well as helping to maintain GBM and glomerular integrity, they are also responsible for phagocytosis of macromolecules trapped in the GBM, and secretion of prostaglandins and cytokines (Vize *et al.*, 2003).

Endothelial cells

During nephron formation endothelial precursors invade the developing glomerulus at the distal portion. The vascular portions of the glomerulus develop from these invading endothelial cells.

The formation process of new blood vessels can take place in two different steps, one is vasculogenesis, or the formation of new blood vessels, and the other is angiogenesis, where new capillaries sprout from pre-existing vessels (Risau,

1998). Both processes contribute to the formation of new vessels in the kidney (Vize *et al.*, 2003).

Vascular endothelial growth factor (VEGF) is required for vasculogenesis and angiogenesis (Risau, 1998). VEGF, with its receptors flk-1 and flt-1, take part in the process of migration, proliferation and differentiation of the renal vasculature (Vize *et al.*, 2003).

Endothelial cells express other endothelial markers such as Platelet endothelial cell adhesion molecule (PECAM) and von Willebrand factor (vWF). PECAM, which is widely used murine endothelial marker, is a member of the immunoglobulin superfamily. PECAM is located in the cell-cell borders of vascular endothelial cells and during inflammatory processes is responsible for the migration of monocytes and neutrophils out of the blood vessels (Redick and Bautch, 1999). vWF is a glycoprotein that mediates platelet adhesion to subendothelium where vascular injury has occurred (Pusztaszeri *et al.*, 2006). Like PECAM, vWF is commonly used as an immunohistochemical marker for endothelial cells (Pusztaszeri *et al.*, 2006).

1.1.2 Tubular markers

Proximal tubular cells

A sudden change in epithelial morphology occurs at the urinary pole of the Bowman's capsule, which demarcates the beginning of the proximal tubules. At this point it is possible to appreciate the transition from simple squamous to simple cuboidal epithelium (Vize *et al.*, 2003). The epithelial cells forming the proximal tubules present a dense brush border of microvilli that enlarge the

absortion area. This large absortion area allows the cells to modify the ultrafiltrate with the resorption of numerous compounds, including proteins, aminoacids, glucose, and creatinine, but mainly water. The brush border of these cells is rich in water channels (Vize *et al.*, 2003) such as channel aquaporin-1 (AQP1), which is expressed in renal proximal tubular cells and the thin descending limb of the loop of Henle (Georgas *et al.*, 2008; Jenq *et al.*, 1999).

Proximal tubular cells play an important role in reabsorption of fluids filtered by the glomerulus as well as most of the filtered glucose, amino acids and bicarbonate (Schnermann *et al.*, 1998). AQP1 null mice present decreased water permeability in the proximal tubule, and defective fluid absorption (Schnermann *et al.*, 1998).

Another protein that is important for the correct functioning of the proximal tubules is the 600-kDa transmembrane glycoprotein, megalin, which belongs to the low-density lipoprotein receptor family (Christensen and Verroust, 2002; Verroust and Christensen, 2002). Megalin is expressed in proximal tubular cells and is highly expressed at the apical surface of the cells. Megalin is able to bind a large number of plasma proteins such as albumin, insulin, prolactin and many others; megalin therefore plays an important role in protein reabsorption in proximal tubules (Christensen and Verroust, 2002; Verroust and Christensen, 2002). Megalin-deficient mice present low molecular weight proteinuria and a deficiency in tubular resorption (Leheste *et al.*, 1999).

The brush border of proximal tubular cells contains membrane-bound enzymes such as alkaline phosphatase, the function of which is related to ion and solute transport (Taub *et al.*, 2002; Vize *et al.*, 2003).

The loop of Henle can be divided in three portions: the thin descending, thin ascending and thick ascending limbs. The thin descending limb is permeable to water and ions while the thin ascending limb is water-impermeable. Aquaporin-1 is expressed in the thin descending limb but is absent in the thin ascending limb (Maunsbach *et al.*, 1997).

Distal tubular cells

Two different portions, the convoluted and the straight, can be identified in distal tubules. Maintenance of the osmotic gradient in the renal medulla seems to be regulated by two tubular fractions: distal straight tubule and the thin ascending limb. Tamm-Horsfall (THP) protein is a 85-kDa glycoprotein localized in the thick ascending limb of the loop of Henle and the early distal convoluted tubule (Chakraborty *et al.*, 2004). The pattern of expression of THP suggests that this protein might have a role in maintaining the medullary osmotic gradient (Kokot and Dulawa, 2000; Vize *et al.*, 2003). THP null mice develop calcium crystal formation and are susceptible to urinary tract infections, suggesting that the specific kidney glycoprotein THP plays an essential role in defence against urinary tract infections and formation of renal stones (Mo *et al.*, 2004).

Collecting duct cells

The collecting ducts are connected to the distal tubules through the connecting segment (Vize *et al.*, 2003). As the collecting ducts approach the papilla they increase in size (Vize *et al.*, 2003). Two main cells types are present in the collecting ducts: principal cells and intercalated cells (Vize *et al.*, 2003). Principal cells are more numerous and are involved in water transport, expressing the water channel aquaporin-2 (AQP2), which is exclusively expressed in collecting ducts (Nielsen *et al.*, 1995; Vize *et al.*, 2003). The intercalated cells can be subdivided into type A, which are acid excreting cells, and type B cells, which secrete bicarbonate into the urine (Alper *et al.*, 1989; Vize *et al.*, 2003).

AQP2 is vasopressin-regulated water channel and has been proved to be essential for regulation of water balance in the kidney (Rojek *et al.*, 2006). AQP2 null mice presented papillary atrophy and increased pelvic space (Ishibashi and Sasaki, 1997; Rojek *et al.*, 2006). These mice displayed severe defects in urine concentration and reduction of the extracellular fluid volume (Rojek *et al.*, 2006). The mice failed to thrive and died before reaching two weeks old.

1.2 Kidney disease

Different insults can damage the kidney leading to acute or chronic renal failure. Poor renal perfusion, tubular damage and obstruction are the main causes of acute renal failure. When acute renal failure does not recover it leads to irreversible damage to the nephron and thus progresses to chronic renal failure. Chronic renal failure is the permanent loss of renal function, and, at some point, may lead to

endstage renal failure. Between 60-160 people per million of the population are affected by ESRF (Brewster *et al.*, 2001).

1.2.1 Acute renal injury

The mammalian kidney has an extraordinary capacity to restore structure and function after toxic or ischæmic acute renal failure (Bacallao and Fine, 1989; Park *et al.*, 1997). After acute renal failure, tubular epithelial cells are desquamated and only the basement membrane remains. The presence of cells and debris causes blockade of the tubular lumen. In addition, the basement membrane itself can be torn, allowing the passage of molecules from bloodstream to the tubular lumen, for example fibronectin, which attachs to cells and debris in the lumen aggravating the obstruction (Bonventre, 2003).

After acute injury the kidney undergoes a repair process in order to repair the damage and restore functionality (Gupta and Rosenberg, 2008). New cells need to be recruited to replace the damaged cells (Gupta and Rosenberg, 2008). The most likely candidates responsible for tubular regeneration are undamaged neighbouring tubular cells, extrarenal cells, or resident kidney stem cells (Gupta and Rosenberg, 2008).

Different groups have shown that bone marrow cells have the potential to migrate and contribute to tubular repair after acute renal injury (Gupta *et al.*, 2002; Gupta and Rosenberg, 2008; Kale *et al.*, 2003; Lin *et al.*, 2003). However, it has been demonstrated that most of the tubular repair occurs by proliferation of renal endogenous cells (Krause and Cantley, 2005; Lin *et al.*, 2005).

Bonventre *et al.* (2003) and Humphreys *et al.* (2008) demonstrated that after acute kidney injury tubular epithelial cells that had survived the damage dedifferentiated, and started to proliferate in order to replace the damaged tubules and restore renal function (Bonventre, 2003). Under physiological circumstances, proximal tubular cells display a low proliferation activity, as shown by proliferative cell nuclear antigen (PCNA) and Ki-67 immunostaining (Bonventre, 2003; Nadasdy *et al.*, 1994). Under normal circumstances this tubular cell proliferation replaces tubular cells lost in urine (Bonventre, 2003). After an ischæmic injury, in which tubular cell death occurs, this proliferation activity dramatically increases in order to replace damaged cells (Bonventre, 2003). Humphreys *et al.* (2008) showed that intrinsic cells displaying epithelial markers are responsible for tubular repair after injury (Humphreys *et al.*, 2008). This study does not exclude the possibility that stem cells play an important role in renal repair nor the possibility that the cells were renal stem cells expressing epithelial markers (Gupta and Rosenberg, 2008). Characteristic markers of undifferentiated renal cells such as vimentin and neural cell adhesion molecule (NCAM) are re-expressed in tubular cells approximately 5 days after injury (Bonventre, 2003). When acute damage is severe enough to require replacement therapies such as dialysis, in-hospital mortality is higher than 50% (Lameire *et al.*, 2005). Acute renal failure seems to be reversible in 5% of cases, however, residual damage in children can cause renal failure in adolescence or adulthood (Lameire *et al.*, 2005).

1.2.2 Chronic kidney disease

Some kidney diseases cause irreversible nephron degeneration. In chronic kidney disease there is a progressive loss of nephrons that results in a gradual loss of renal function (Kriz and LeHir, 2005). It has been shown that in humans and animal models, most of the diseases that progress to chronic renal failure start with glomerular damage. Independently of whether the original disease persists or not, there is a progressive loss of nephrons in chronic renal disease and loss of renal function as a consequence. In chronic renal disease, normal nephrons are usually found close to damaged nephrons. Two hypotheses have tried to explain how damaged nephrons affect surrounding healthy nephrons. Protein overload in a particular tubule causes a localized interstitial inflammation that damages the tubule and also the neighbouring ones. The second possibility is that as a consequence of nephron loss the remaining nephrons need to compensate for the lack of functionality. Compensatory mechanisms, such as hypertrophy, result in hyperfiltration and protein leakage leading to glomerular sclerosis. These nephrons do not appear to have any potential to recover and as a consequence are permanently lost (Kriz and LeHir, 2005).

Transplantation and dialysis are the only available treatments for ESRF, and without them ESRF would lead to death. However, the shortage of organs available for transplantation and the increasing number of patients on dialysis that need a new kidney make it necessary to search for novel alternatives to deal with renal failure (Brewster *et al.*, 2001; Brodie and Humes, 2005). The relatively long time window between the onset of symptoms of chronic renal failure and the

progression of the disease to ESRF provides an ideal opportunity for intervention with a regenerative stem cell therapy.

1.3 Kidney development

1.3.1 Overview of mammalian kidney development: the pronephros, mesonephros and metanephros

The adult kidney is part of the urogenital system, which is mainly derived from the intermediate mesoderm of the mid-gestation mouse embryo (Saxen, 1987; Torres *et al.*, 1995). The intermediate mesoderm is located between the paraxial mesoderm and the lateral plate mesoderm.

The coordinated differentiation of two different tissues derived from intermediate mesoderm gives rise to the urogenital system. One of these tissues is the ductal epithelium, which is responsible for generating the genital tracts, ureters and renal collecting duct system, and the other is the nephrogenic mesenchyme, which forms the nephrons of the mesonephric and metanephric kidney (Torres *et al.*, 1995).

The formation of the nephric duct is the first epithelial conversion that takes place during kidney development (Vize *et al.*, 2003). The nephric duct, also called Wolffian or mesonephric duct, extends towards the caudal portion of the embryo bilaterally until it reaches the cloaca (Lanza *et al.*, 2006). As the nephric duct extends caudally, the three embryonic kidneys, the pronephros, mesonephros and metanephros, develop sequentially (Figure 1-4) (Saxen, 1987; Torres *et al.*, 1995).

The pronephros develops along the anterior part of the nephric duct and is comprised of a linear array of tubules that empty into the Wolffian duct (Saxen, 1987; Torres *et al.*, 1995). The pronephros is the most primitive of the three kidneys and in mammals the pronephric tubules are very rudimentary (Saxen, 1987; Vize *et al.*, 2003). Fish, including all teleosts, such as zebrafish (*Danio rerio*), and more primitive groups, such as lampreys and hagfish, and amphibians have well developed pronephroi that are only functional in larval stages and are replaced by the mesonephros in adult stages (Saxen, 1987; Vize *et al.*, 2003).

In mammals, the mesonephros is the second kidney to form during development. The number of mesonephric tubules in mammals varies from 30 to 50 depending on the species (Saxen, 1987). Mesonephric nephron formation starts with the development of a mesenchymal condensate giving rise later on to renal vesicles and S-shaped bodies that connect with the Wolffian duct (Saxen, 1987). Different portions of the mesonephric nephron can be distinguished, such as the mesonephric corpuscle, proximal tubule, distal tubule, and collecting tubule. The maturation of the mesonephric nephrons closely resembles the maturation of nephrons in permanent kidneys. However, there are some noticeable differences; for instance the mesonephric nephrons lack a juxtaglomerular apparatus, the fenestration of capillaries is poor, and the glomerular basement membrane is immature in comparison with metanephric nephrons (Saxen, 1987).

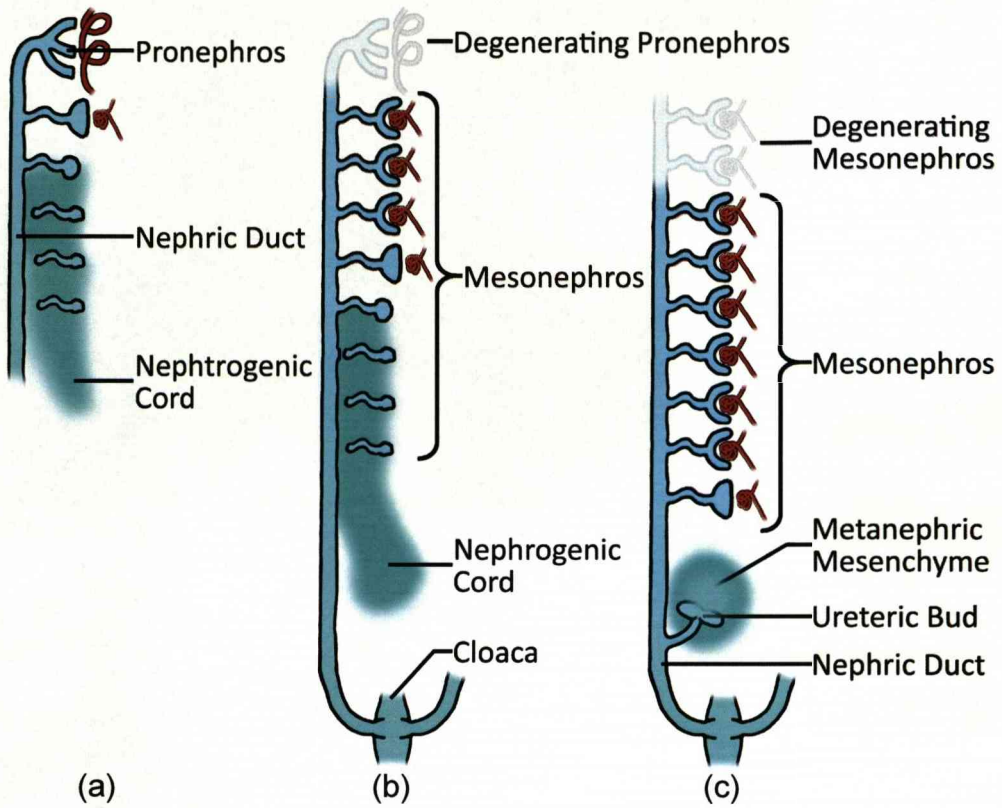


Figure 1-4 Diagram of the development of the vertebrate kidney showing (a) pronephros, (b) mesonephros and (c) metanephros (from Ashley Sawle with permission under the terms of the Creative Commons Attribution Sharealike 3.0 license, http://en.wikipedia.org/wiki/File:Development_of_embyonic_nephrons.png).

The pronephros and mesonephros are transient structures in mammals that are later replaced by the metanephros, or adult kidney, which develops at the caudal end of the nephric duct (Lanza *et al.*, 2006). Before birth, the embryonic kidneys and the adult kidneys are not essential for survival in many mammals, as has been proved by the live birth of many humans completely lacking kidneys (Vize *et al.*, 2003).

The adult kidney starts to form when a population of cells known as metanephric mesenchyme (MM) becomes demarcated in the caudal part of the intermediate mesoderm. MM is situated about 1–200 μm from the Wolffian duct and its

demarcation takes place at embryonic day 10 (E10) in mice and E30 in humans (Vize *et al.*, 2003). The MM secretes some growth factors, such as GDNF and HGF, that induce the Wolffian duct to sprout and form the ureteric bud (UB), which elongates and invades the nearby MM (Saxen, 1987).

1.3.2 Ureteric bud development

After the UB invades the MM at E11 in mouse, the ends of the bud undergo a series of terminal bifurcations. During the first bifurcation, the UB divides into two branches that grow in opposite directions with an angle of approximately 180°, giving rise to a T-shape structure. The process of branching continues with gradually decreasing angles (Lanza *et al.*, 2006; Saxen, 1987). The pelvis and calyces are formed by remodelling of the first 6 to 10 branch generations of the UB while the final 6 to 10 form the collecting ducts (Woolf *et al.*, 2004).

1.3.3 Nephron formation

The nephron is the basic functional unit of the kidney (Vize *et al.*, 2003). Signals required for ureteric bud growth and branching are secreted by MM, while the UB provides the MM with the essential signals to condense around the ureteric bud tips and differentiate into the epithelium of the developing nephrons (Figure 1-5) (Vize *et al.*, 2003).

Cells from metanephric mesenchyme condense around the tips of the ureteric bud and differentiate to a polarized epithelium forming the renal vesicle. The renal vesicle elongates and forms the comma- and then the S-shaped body. In the S-shaped body, the most distal portion from the UB forms the glomerular

epithelium comprised of visceral epithelium and the parietal epithelium of the Bowman's capsule. Endothelial precursors invade this distal portion of the S-shaped body. The proximal end of the S-shaped body fuses with the ureteric bud and forms the distal tubule. The proximal tubule precursors are located between the proximal and distal parts of the S-shaped body. As the immature nephron continues to develop, the proximal tubules form a convoluted mass of epithelium which grows into the medullary area giving rise to the descending and ascending limbs of Henle's loop (Vize *et al.*, 2003). Finally the cells of the visceral epithelium, also known as podocytes, and the endothelial cells interact to form the filtration unit of the nephron. The capillary tuft is formed by the endothelial cells forming the capillaries and the mesangial cells that are highly specialized smooth muscle cells (Lanza *et al.*, 2006).

1.3.4 Genes involved in kidney development

Metanephric kidney formation starts when MM demarcates in the intermediate mesoderm approximately at E10 (times given are for mouse development). MM expresses Pax-2 and WT1, two transcription factors that play an important role in kidney development. Another gene expressed in metanephric mesenchyme that has proved to be essential for ureteric bud invasion is Sall1 (Nishinakamura *et al.*, 2001).

Metanephric mesenchyme secretes glial-derived neural growth factor (GDNF) that interacts with the nearby Wolffian duct, which expresses the GDNF receptor c-Ret and its co-receptor GFR-1α (Pachnis *et al.*, 1993). As a result of these interactions the ureteric bud is induced to sprout at about E10.5; it then grows and

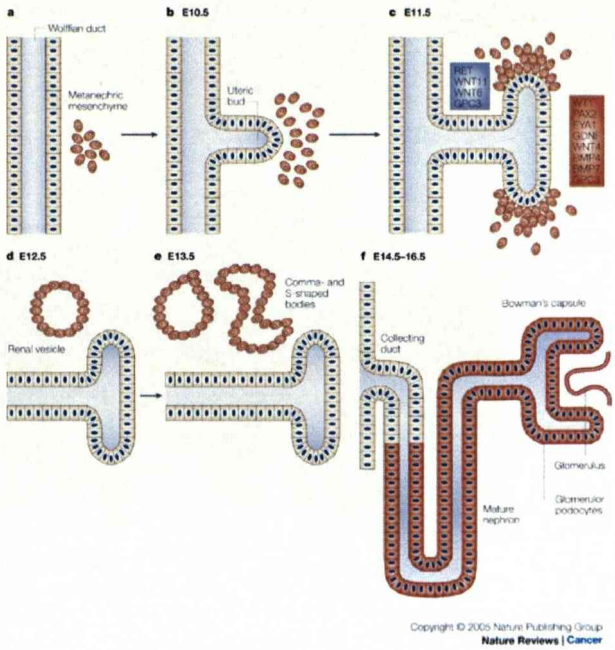


Figure 1-5 Diagram showing reciprocal induction of metanephric mesenchyme and ureteric bud during kidney development. (a) MM demarcation. (b-c) Condensation of MM around UB tips. Mesenchymal to epithelial transition, (d-e) Formation of comma shaped body and S-shaped body formation, (f) Tubule maturation. Nephron and convoluted tubule formation. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer (Rivera and Haber, 2005), copyright (2005)

invades the metanephric mesenchyme at approximately E11 (Vize *et al.*, 2003). GDNF/c-Ret signaling is required for growth and branching of the UB within the MM, so it continues to be expressed once the ureteric bud has invaded the metanephric mesenchyme (Davies and Fisher, 2002; Pachnis *et al.*, 1993). The transcription factor Emx-2 is required for the UB to continue expressing c-Ret after metanephric mesenchyme invasion (Pachnis *et al.*, 1993).

As the ureteric bud grows and branches, it secretes some factors such as TGFα, TIMP-2, EGF and FGF-2 that prevent the metanephric mesenchyme cells from undergoing apoptosis (Coles *et al.*, 1993).

Nephron formation starts approximately at E11.5-12 when groups of cells in the metanephric mesenchyme expressing Pax-2, WT1, NCAM and other proteins condense around the tips of the ureteric bud (Vize *et al.*, 2003).

The ureteric bud induces surrounding mesenchyme to form nephrons. Many different signals secreted by UB seem to induce the metanephric mesenchyme to undergo nephrogenesis.

Perantoni *et al.* (1995) showed that FGF-2 can mimic early events in nephron formation, inducing MM to condensate in the absence of an inductor tissue, however, no epithelial conversion was achieved. Another factor that seems to be relevant in nephrogenesis is leukemia inhibitory factor (LIF). LIF is secreted by UB, and in combination with FGF-2 induces nephron formation in 7 days (Barasch *et al.*, 1999). A combination of FGF-2, LIF and TGF-β induce nephron formation in isolated mesenchyme in 72 hours as *in vivo* (Plisov *et al.*, 2001).

When cells from metanephric mesenchyme condense around the tips of the ureteric bud they express Wnt4 (Kispert *et al.*, 1998). Wnt4 expression is required for mesenchymal to epithelial transition of metanephric mesenchyme, and is therefore necessary for nephrogenesis to continue (Kispert *et al.*, 1998; Stark *et al.*, 1994).

Another important molecule in controlling morphogenesis of the kidney is bone morphogenic protein 7 (BMP-7). This morphogenic protein is secreted by metanephric mesenchyme and ureteric bud during kidney development (Vukicevic *et al.*, 1996). The effect of BMP-7 in nephrogenesis seems to be dose dependent. Low concentrations of BMP-7 seem to promote ureteric bud

branching whereas high concentrations seem to inhibit it. This may be a mechanism for balancing branching and nephrogenesis during development (Vukicevic *et al.*, 1996).

1.3.5 Pax-2

Pax-2 encodes a paired box-containing transcription factor that plays an important role during kidney development (Dressler, 1996; Vize *et al.*, 2003). Pax-2 expression in mouse is restricted to developing nervous and excretory systems (Nornes *et al.*, 1990; Vize *et al.*, 2003). During kidney development, Pax-2 is expressed in ductal epithelium and nephrogenic mesenchyme during development of the pronephros, mesonephros, and metanephros (Dressler *et al.*, 1990; Torres *et al.*, 1995).

During renal development, Pax-2 is observed for the first time in the pronephric duct at about 12–13th somite, and as development progresses, Pax-2 is observed in Wolffian duct ~E9, and in the pronephric and mesonephric tubules (Dressler *et al.*, 1990; Vize *et al.*, 2003). Pax-2 is expressed in metanephric mesenchyme prior to ureteric bud invasion (Vize *et al.*, 2003), and, as kidney development progresses, expression is up-regulated in condensed metanephric mesenchyme, renal vesicles, comma-shaped bodies, and the distal portion of the S-shaped body (Dressler, 1996; Dressler *et al.*, 1990; Dressler and Douglass, 1992).

Homozygous Pax-2 mutant mice lack kidneys, ureters, and a genital tract (Torres *et al.*, 1995). In these mutants, ureteric bud formation does not take place (Torres *et al.*, 1995). These observations seem to show the crucial role of Pax-2 in early kidney development. Furthermore, Pax-2 expression seems to be essential for

early epithelial differentiation as shown by its expression pattern during normal kidney development (Dressler, 1996; Rothenpieler and Dressler, 1993). Low Pax-2 protein levels lead to failure of the MM to aggregate and undergo morphological changes to nephron formation (Rothenpieler and Dressler, 1993).

Pax-2 is associated with cell proliferation and there is little expression observed in mature kidneys, with the exception of tubular regeneration as a consequence of kidney damage, or some renal tumors, such as renal cell carcinoma or Wilm's tumor (Dressler and Douglass, 1992; Gnarra and Dressler, 1995; Imgrund *et al.*, 1999).

1.3.6 WT1

Wilms' tumor suppression gene (WT1) encodes a zinc finger transcription factor (Yokoo *et al.*, 2008). Initially WT1 was identified as the gene involved in Wilms' tumor. Wilm's tumor is an embryonic kidney neoplasia characterized by undifferentiated mesenchymal cells, poorly organized epithelium and surrounding stromal cells (Vize *et al.*, 2003). Expression of WT1 in mouse embryo is first observed at E9.0 in the intermediate mesenchyme lateral to the coelomic cavity (Armstrong *et al.*, 1993). A few hours later WT1 is expressed in differentiating heart mesothelium and in early mesonephric tubules (Armstrong *et al.*, 1993). WT1 is expressed in uninduced MM approximately at E11, and by E12.5 WT1 expression is increased in the condensed MM of the developing metanephros (Armstrong *et al.*, 1993). One day later, at about E13.5, WT1 is observed in nephrogenic condensations. As kidney development progresses at about E20

WT1 is restricted to glomeruli (Armstrong *et al.*, 1993). WT1 is not expressed in Wolffian duct or ureteric bud (Kreidberg *et al.*, 1993).

It has been demonstrated that WT1 expression is essential for early kidney development. WT1 null mutant mice completely lack kidneys (Kreidberg *et al.*, 1993; Vize *et al.*, 2003). In these mutants the ureteric bud never invades the metanephric mesenchyme, and without bud invasion MM quickly becomes apoptotic (Kreidberg *et al.*, 1993; Vize *et al.*, 2003).

Davies *et al.* (2003) showed that WT1 is not only required in early kidney development, but also plays an essential role in nephron formation. Davies *et al.* (2003) used small interfering RNA (siRNA) to suppress WT1 at different stages of kidney development, and showed that when WT1 is suppressed in MM at pre-epithelial stage, cells proliferate abnormally and do not differentiate into nephrons (Davies *et al.*, 2003).

1.3.7 Comparison of Pax-2 and WT1 expression during kidney development

WT1 is expressed at low levels in uninduced metanephric mesenchyme where Pax-2 is also expressed (Ryan *et al.*, 1995; Vize *et al.*, 2003). By E12 when ureteric bud has invaded metanephric mesenchyme and has branched 4 to 8 times Pax-2 is highly expressed in condensed mesenchyme where WT1 is barely detected (Ryan *et al.*, 1995). As nephrogenesis progresses, Pax-2 is maintained in condensing mesenchyme and comma-shaped bodies, and expression starts to decline in the proximal loop of the S-shaped bodies (Dressler and Douglass, 1992; Ryan *et al.*, 1995). As this happens, WT1 is expressed in the cells of the

proximal portions of the S-shaped bodies where Pax-2 is downregulated. High levels of WT1 are present in the cells forming the glomerular epithelium (Ryan *et al.*, 1995). High levels of Pax-2 and WT1 are not co-expressed during kidney development (Ryan *et al.*, 1995).

1.3.8 Sall1

Sal like 1 gene (Sall1) is a mammalian homologue of the *Drosophila* region-specific homeotic gene *spalt (sal)* (Nishinakamura *et al.*, 2001). During kidney development Sall1 is expressed in metanephric mesenchyme. Nishinakamura *et al.* (2001) showed that Sall1 expression in metanephric mesenchyme is required for ureteric bud invasion. As a result of homozygous deletion of Sall1 ureteric bud failed to invade the metanephric mesenchyme (Nishinakamura *et al.*, 2001). Cells strongly expressing Sall1 have the potential to give rise to cell types in the nephron such as podocytes, and proximal and distal tubule cells (Osafune *et al.*, 2006).

1.3.9 GDNF

Glial cell line-derived neurotrophic factor (GDNF) is a glycosylated, disulfide-bonded homodimer that is a distant member of the transforming growth factor-β (TGF-β) superfamily (Lin *et al.*, 1993).

GDNF expression is first observed in nephrogenic cord in mouse embryos at E8.5 (Sanchez *et al.*, 1996). GDNF expression is later on restricted to metanephric mesenchyme approximately at E10.5 (Sanchez *et al.*, 1996).

GDNF signal is transduced by a receptor complex formed by the GDNF receptor alpha (GDNFR-α) and the cRet receptor tyrosine kinase (Sainio *et al.*, 1997; Vize *et al.*, 2003). cRet is expressed in ureteric bud epithelium (Pachnis *et al.*, 1993) and is autophosphorilated once GDNF binds (Sainio *et al.*, 1997).

GDNFR-α, one of the GDNF receptors, is expressed in metanephric mesenchyme and ureteric bud, however, it has been shown that GDNF binds exclusively to the tips of the ureteric bud branches where cRet receptor is expressed. In summary GDNF is involved in kidney development where it promotes ureteric bud outgrowth from Wolffian duct and is essential for ureteric bud branching (Sainio *et al.*, 1997).

1.4 Stem cells in kidney repair

Stem cells are specialized cells with the ability of self-renewal. Self-renewing potential is the property of a cell to generate at least one daughter cell identical to the original cell (Lanza *et al.*, 2006). Self-renewing allows stem cells to maintain the stem cell pool. Another property of stem cells is the potential to differentiate into a variety specialized cell types. Stem cells change to lineage commitment and give rise to progenitor, precursors and differentiated cells. When stem cells differentiate they acquire morphological, phenotypical, and functional characteristics of a specific cell type (Lanza *et al.*, 2006).

Owing to their potential to give rise to specialized cell types, stem cells could be used to replace damaged tissues. According to Yokoo *et al.* (2008) two approaches could be used in order to repair kidney damage when renal structure

is not completely lost. Resident stem cells in the kidney could be stimulated in order to make them differentiate to specific renal types and replace the lost tissue to restore functionality. A second approach could be to expand stem cells *in vitro* and differentiate them into the specialised cells required in the damaged kidney. However, if kidney structure has suffered extensive damage the whole kidney structure would need to be developed (Yokoo *et al.*, 2008).

Different stem cells could be used to replace cells in damaged kidneys. It has been shown that stem cells from bone marrow have the potential to generate differentiated kidney cells such as mesangial cells (Imasawa *et al.*, 2001), podocytes (Poulsom *et al.*, 2001) and tubular cells (Gupta *et al.*, 2002; Kale *et al.*, 2003).

Another approach for the stem cell therapy is the use of embryonic stem cells. Different groups have tried to generate kidney cells from embryonic stem cells (ES) (Bruce *et al.*, 2007; Kim and Dressler, 2005; Vigneau *et al.*, 2007). Embryonic stem cells are pluripotent cells, which mean that they have the potential to give rise to somatic cell lineages in the body (Lanza *et al.*, 2006). It has been shown that mouse embryonic stem cells have the potential to generate cells expressing renal specific markers *in vitro* and *in vivo* (Bruce *et al.*, 2007; Kim and Dressler, 2005; Vigneau *et al.*, 2007). However, embryonic stem cells injected *in vivo* can generate tumours, which may limit their use in therapies to replace damaged tissues (Gomez and Thorgeirsson, 1998; Roufosse and Cook, 2008).

The third alternative to regenerate the tissue lost as a consequence of damage is the use of tissue specific stem cells (Ricardo and Deane, 2005). The advantage of using tissue stem cells is that they could be isolated from the patient, and therefore rejection would not be an issue when they were used later on to treat that patient. It is believed that tissue stem cells have some differentiation commitment, and that they may give rise to cells from their tissue of origin making the task of generating specific cell for that organ easier and avoiding formation of unwanted cells from other tissues (Bussolati *et al.*, 2008).

1.5 Evidence of tissue specific stem cells in adult kidney

Isolation of kidney stem cells has proved to be a difficult task due to the lack of a definitive marker characteristic of these tissue specific cells (Gupta and Rosenberg, 2008). A variety of different methods have been used in the attempt to isolate stem cells from kidneys in human and rodents including isolation using bromodeoxyuridine, identification of side populations by flow cytometry, selection using specific stem cell markers, and enrichment using selective culture conditions.

1.5.1 Bromodeoxyuridine labeling

Tissue stem cells *in vivo* present a low cycling rate. This property has been used to identify stem cells in kidney. When cells are labelled with bromodeoxyuridine (BrdU), it is incorporated into the DNA of the cells during DNA synthesis. Only slow cycling cells will retain the labelling.

When Oliver *et al.* (2004) used this labelling technique to find kidney stem cells in rodents, they found a population of slowly cycling cells in the renal papilla retaining BrdU. These BrdU retaining cells disappeared from the papilla only after renal injury, suggesting a role in renal repair. In the papilla the majority of these BrdU retaining cells were located in the interstitium, but some were also located in the epithelium of tubules. Cells from the papilla were expanded *in vitro* and some of them, as previously described for metanephric mesenchyme cells (Oliver *et al.*, 2002), were found to co-express epithelial and mesenchymal markers. Another interesting property of these papillary renal cells in culture was that they were able to form aggregates or spheres; this is a characteristic of embryonic stem cells and adult stem cells in culture. When the culture conditions of clones expanded from single cells were changed the cells changed phenotype. Cells expanded from single clones gave rise to a heterogenic population of cells and some of these cells expressed neuronal markers. These results suggested that renal papillary cells isolated by Oliver *et al.* (2004) were a pluripotent population able to generate different specialized cells.

Maeshima *et al.* (2006), on the other hand, described a population of low rate cycling cells (LRC) present mostly in proximal tubules, but also in distal tubules and collecting ducts, by BrdU labelling. They proposed that these low cycling cells present in the tubules, are an important contribution to regeneration after ischæmia.

Hoechst 33342 is fluorescent nuclear dye that is highly specific for thymine. In cells labelled with BrdU the thymidine has been substituted by the BrdU and thus

these cells will only stain relatively weakly with Hoechst 33342. Maeshima *et al.* (2006) used this property to isolate the LRC population from adult kidney by fluorescence-activated cell sorting (FACS) and studied their properties (Maeshima *et al.*, 2006). They were able to expand these cells in culture, and showed that their proliferation and differentiation properties were affected by factors present in conditioned media from a metanephric mesenchyme line. When the cells were grown in collagen they formed cysts and tubular structures. They also integrated into structures when injected in developing kidneys (Maeshima *et al.*, 2006).

Although both experiments were done in rats, in the case of Maeshima *et al.* (2006; 2003) the low cycling cells were located in the tubules while the low cell cycling cells in the study of Oliver *et al.* were located in the interstitium of the papilla (Oliver *et al.*, 2004). Maeshima *et al.* (Maeshima *et al.*, 2006) proposed that these differences could be due to differences in the protocol followed for the BrdU labelling, and also the age of the animals used (Maeshima *et al.*, 2003; Maeshima *et al.*, 2006; Oliver *et al.*, 2004).

1.5.2 Side populations

Goodell *et al.* (1996) proposed a new method for enriching hematopoietic stem cells from bone marrow. They stained murine bone marrow cells with Hoechst 33342 and identified a small cluster which displayed different staining patterns when analysed by FACS. This side population (SP) displayed phenotypic markers of hæmatopoietic stem cells.

This approach has also been used to isolate kidney stem cells in rodents. Iwatani *et al.* (2004) isolated SP cells from adult rat kidneys. These cells did not seem to have any regenerative potential or engraftment when they were injected in rats with induced glomerulonephritis, nor did they demonstrate renal potential *in vitro*.

Hishikawa *et al.* (2005) isolated a side population in mouse kidney. The SP cells expressed musculin/MyoR, which was absent in the non-SP cells. These cells were located in the interstitial space. Injection of these cells to kidneys improved renal function in acute renal failure.

Challen *et al.* (2006) also isolated SP cells from adult mouse kidneys. These cells were located in proximal tubules and had the potential to differentiate *in vitro* to different lineages. The SP isolated in this study did not express musculin/MyoR. When these cells were injected after acute kidney injury, they seemed to improve renal function without significant tubular integration, suggesting that they may have a role in promoting repair (Challen *et al.*, 2006).

1.5.3 Stem cells markers

Dekel *et al.* (2006) reported the existence in adult kidney of a population of non-tubular cells expressing stem cell antigen-1 (Sca-1). Sca-1 is a cell surface marker for hematopoietic stem cells and tissue stem cells (Dekel *et al.*, 2006; Kondo *et al.*, 2003). Sca-1 positive cells were selected from the non-tubular fraction of adult mouse kidneys. Clones were derived from single cells and could be differentiated into myogenic, osteogenic, adipogenic, and neural cells. They also differentiated to a tubular phenotype when injected into renal parenchyma after

acute renal injury (Dekel *et al.*, 2006). These results suggested that a Sca-1 population present in adult kidney may be multipotent stem cells (Dekel *et al.*, 2006).

1.5.4 Culture conditions

Another approach has been to isolate stem cells using selective culture conditions that favour the survival of stem cells over differentiated cells (Gupta *et al.*, 2006). Gupta *et al.* (2006) plated disaggregated cells from rat kidney, and used expansion media that had previously been proved to support expansion of stem cells from human bone marrow (Jiang *et al.*, 2002). They assumed that these culture media would promote expansion of potential stem cells present in rat kidney rather than differentiated cell types. These rat renal stem cells could self-renew, were clonogenic, and expressed undifferentiated markers, such as vimentin, Oct4, Pax-2 and CD90, but no differentiated markers. They could also differentiate *in vitro* to cells of all three germ layers, and could integrate and differentiate *in vivo* into renal tubules after acute renal damage.

A similar approach was used by Kitamura *et al.* (Kitamura *et al.*, 2005), who dissected different portions of the nephrons from a rat kidney to find that the proximal tubules contained a fraction of the cells with high proliferation potential. Plating the cells at cloning density resulted in only a few cells forming colonies. From these cells they generated a cell line with high proliferation potential, potential to generate differentiated renal epithelial cells, and the ability to integrate in tubular epithelium after acute kidney damage.

1.5.5 CD133 expressing cells in human kidney

Other evidence of the existence of renal resident stem cells in the adult human kidney was proposed by Sagrinati *et al.* (2006). They isolated another population of resident renal stem cells using CD133 and CD24 in the epithelium of the Bowman's capsule. These cells had properties of self-renewal and clonogenicity, and the capacity to differentiate *in vitro* into renal cells such as proximal and distal tubular cells, adipogenic and osteogenic cells, and neuronal cells. Intravenous injection of these cells into immunocompromised SCID mice that had acute renal failure resulted in regeneration of tubular structures (Sagrinati *et al.*, 2006).

Bussolati *et al.* (2005) showed that the cortex of human adult kidney contained a population of cells that express CD133 suggesting that they were renal stem cells. In a series of experiments they demonstrated that these cells were clonogenic and had the capability to self-renew and to differentiate *in vitro* into epithelial and endothelial lineages. This further reinforced the possibility that these cells represented an extant population of renal stem cells in adult kidney. These cells were also shown to co-express the transcription factor Pax-2, which plays a crucial role in urogenital development (Vize *et al.*, 2003). Pax-2 is also highly expressed in the nephrogenic zone, but decreases when the cells begin to differentiate (Ryan *et al.*, 1995).

1.6 Animal model to study kidney stem cells

The fact that healthy human organs are used for transplantation makes it necessary to search for alternatives to study stem cells in organs. In humans nephrogenesis is complete before birth, by approximately during 38 week of gestation, however, in mice nephrogenesis continues after birth (Pierre *et al.*, 2008). This makes the mouse a convenient model for understanding the role of stem cells in forming nephrons. Furthermore, working with mice allows a complete study of the whole kidney, whereas working with human kidney is only possible with small biopsy samples made available for study.

1.7 Conditions to grow stem cells *in vitro*

Adhesion to an extracellular matrix is essential for the majority of the cells in order to survive and proliferate (Xu *et al.*, 2001). Various studies have suggested that embryonic stem cells require fibroblast feeders in order to maintain their undifferentiated and pluripotent status *in vitro* (Thomson, 1998). However, several groups have successfully replaced fibroblast feeder cells with different extracellular matrix components such as fibronectin, laminin, collagens, or Matrigel™ (Klimanskaya *et al.*, 2005; Rosler *et al.*, 2004; Xu *et al.*, 2001).

The feeders not only provide the stem cells with an adequate substrate to adhere to, but also secrete soluble factors to the media that may be important to maintain stem cells properties (Xu *et al.*, 2001). When fibroblast feeders are substituted for deoxycholate treated fibroblast feeder cells or extracellular matrix proteins, the

essential factors released by the feeders can be supplied using conditioned medium collected from fibroblasts (Rosler *et al.*, 2004; Xu *et al.*, 2001).

1.7.1 Substrates

Matrigel

Matrigel™ is a soluble mix of extracellular matrix components produced by BD Biosciences (Franklin Lakes, New Jersey). The main components of Matrigel™ are laminin followed by collagen IV, heparan sulfate proteoglycan and entactin (Ohashi K. *et al.*, 2006; Xu *et al.*, 2001). Xu *et al.* (2001) demonstrated that human embryonic stem cells can be grown undifferentiated on Matrigel™ coated dishes.

Feeder cells

It is possible to grow embryonic stem cells (ES) in the absence of feeder cells, however, most of the research groups working with stem cells rely on the feeders to provide factors that promote proliferation and support the undifferentiated status of the stem cells.

The most commonly used feeders in stem cell cultures are primary cultures of mouse embryonic fibroblast (MEFs) or STO mouse fibroblast cell line. MEFs are a reliable and a potent source of feeders, however, as primary cells they have a limited life span and they need to be continuously replaced with fresh stocks (Hogan *et al.*, 1994; Park *et al.*, 2004; Wang *et al.*, 2005).

STO are a thioguanine- and oubain-resistant subline of SIM mouse fibroblast isolated by Dr. Bernstein (Martin and Evans, 1975). STO cell lines have been used as feeders to grow ES cells (Hogan *et al.*, 1994).

Sodium deoxycholate treated STO feeders

Fibroblasts grown *in vitro* secrete a pericellular matrix that is rich in glycosaminoglycans such as hyaluronic acid, heparan sulphate, fibronectin, and procollagen types I and III. These components of the matrix are involved in cell adhesion (Hedman *et al.*, 1978).

Hedman *et al.* (1979) developed a method to isolate the pericellular matrix of cultured fibroblast by treating the fibroblast with a sodium deoxycholate detergent solution. Klimanskaya *et al.* (2005) used this technique to remove fibroblast from culture and retain the extracellular matrix in order to use it as a substrate to grow stem cells. Human embryonic stem cells (hES) grown in deoxycholate treated feeders maintained normal karyotype, and expression of pluripotency markers such as Oct-4, SSEA-3, SSEA-4, Tra-1-60, and alkaline phosphatase (Klimanskaya *et al.*, 2005). After six months undifferentiated in culture, the hES were able to give rise to cells from the three different germ layers. This method provides a substrate that allows the growing of stem cells undifferentiated and free of fibroblast feeders that could interfere in the analysis of the cells.

Fibronectin

The extracellular matrix (ECM) lies in the intercellular space providing physical support to tissues and organs (Ghajar and Bissell, 2008). The ECM is formed by a heterogeneous mix of water, saccharides, and protein components that can be classified into four groups: collagens, proteoglycans, non-collagenous glycoproteins and elastins. The ECM not only provides support for the cells in tissues, but it also affects cell functions by two mechanisms. Firstly, ECM functions as storage for molecules such as growth factors, cytokines, chemokines, and enzymes. Secondly, ECM proteins affect cell adhesion and migration by interacting with cellular receptors (Aszodi *et al.*, 2006).

Fibronectin is an adhesive glycoprotein of high molecular weight present in the extracellular matrix. Fibronectin regulates some cell functions by interacting with other ECM molecules, integrins, and syndecans (Aszodi *et al.*, 2006).

Most of the groups that isolated kidney stem cells plated the cells onto fibronectin after isolation of the cells (Bussolati *et al.*, 2005; Gupta *et al.*, 2006; Oliver *et al.*, 2004; Sagrinati *et al.*, 2006).

1.7.2 Culture medium for expanding stem cells

The basal medium that have been used by most groups to grow embryonic stem cells consists of Dulbecco's modified Eagle's medium (DMEM) with high glucose (4500 mg/l) and addition of 1 mM of sodium pyruvate, 1 mM glutamine, 0.1 mM β-mercaptethanol and 1% non-essential aminoacids (Bussolati *et al.*, 2005; Gupta *et al.*, 2006; Peister *et al.*, 2004; Sagrinati *et al.*, 2006). Foetal calf serum is also added to this basal medium in a range of 10-20% (Hogan *et al.*,

1994). However, to culture tissue stem cells various different basal media have been used containing α-modified minimum essential medium (αMEM), Dulbecco's modified Eagle's medium (DMEM), IMDM, RPMI-1640, DMEM-LG, or MCDB-201, and a standard protocol has not become established (Bussolati *et al.*, 2005; Gupta *et al.*, 2006; Peister *et al.*, 2004; Sagrinati *et al.*, 2006).

1.8 Aims

The possibility of treating kidney disease with a regenerative or repair therapy based on adult stem cells taken from the patient promises a valuable alternative to transplant. Recent developments have shown that this is feasible for organs such as the bladder and lungs. The kidney represents a fundamentally more complex situation. The first step in developing such a therapy would be to isolate a population of stem cells capable of giving rise to the wide variety of cell types required to generate a functional nephron.

Recent studies have indicated that the existence of an extant population of kidney stem cells in adult human kidney is a possibility. Unfortunately working with human kidney samples is problematic and in the first instance an alternative model species is preferred. The mouse provides a suitable alternative, and has the advantage that nephrogenesis continues after birth, meaning that accessing tissue that is undergoing kidney development is relatively simple.

The following study was aimed, in the first instance, at investigating the existence of stem cells in postnatal mouse kidney. Firstly, to study how their prevalence

and location alters during kidney development and later to determine whether or not it would be possible to identify a population of stem cells in mature kidney.

Following identification of stem cells in the post-natal kidney, the next step in the study was to isolate these kidney stem cells in order to further study their properties. Magnetic-activated cell sorting (MACS) was the preferred technique to separate the population of stem cells from the rest of the cells in the kidney. A series of lectins was screened in order to identify a suitable cell surface marker to bind the stem cells and separation using MACS was also performed.

As a variety of substrates and media have been successfully used to culture stem cells a study was undertaken, following isolation of the cells, to optimize the culture condition to promote expansion and self-renewal. Under these optimized conditions the putative stem cells were then assessed to determine whether they exhibited other properties of stem cells, such as the ability to form clones and the ability differentiate to specific renal or extrarenal cell types.

Chapter 2: Materials and Methods

2.1 WT1-Cre/ROSA26 transgenic mice

Mice that were transgenic for the construct Rosa26R-lox-STOP-lox-LacZ were crossed with transgenic mice that expressed Cre recombinase under control of the WT1 promoter (Figure 2-1) (Wilm *et al.*, 2005). In offspring that were transgenic for both constructs the activation of the WT1 promoter would result in the expression of Cre recombinase, which would then permanently remove the stop sequence between the lox sites in the Rosa26R-lox-STOP-lox-LacZ construct. This would cause transcription of the LacZ reporter gene resulting in expression of β-galactosidase. The expression of β-galactosidase would continue even after WT1 expression had ceased allowing cells that had previously, but no longer, expressed WT1 to be identified. Offspring transgenic for both constructs were selected and their kidneys were isolated 7 days after birth. Transgenic mice strains were created by Bettina Wilm, University of Vanderbilt, who then supplied the 7 day old kidneys (Wilm *et al.*, 2005).

2.2 Kidney Dissection

2.2.1 Embryonic kidney rudiment

Pregnant CD-1 mice (Charles River, UK) were sacrificed at 11.5 days following timed mating with CO_2 and/or cervical dislocation. Embryos were removed from uteri of pregnant mice at embryonic day 11.5. Embryos were placed in a dish containing dissecting media (see Kidney rudiment culture medium, page 81). Using a 25 gauge hypodermic needle (Becton Dickinson, New Jersey, USA) the

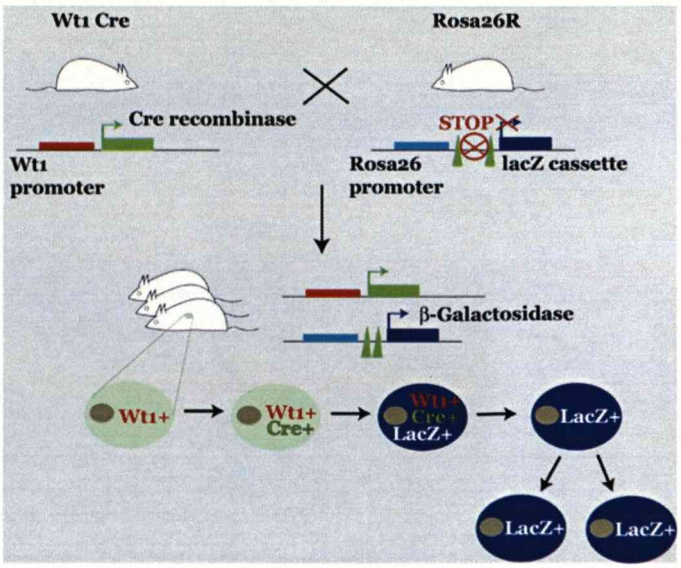


Figure 2-1 Schematic illustrating WT1-Cre/ROSA26R transgenic strategy. Mice transgenic for R26Rosa-lox-STOP-Lox-LacZ in which a stop sequence is separating the reporter gene LacZ from the Rosa26 promoter were crossed with mice transgenic for WT1-Cre recombinase. Offspring carrying both constructs were selected. In cells that express WT1, cre-recombinaseis expressed, causing recombination of the lox sites and removal of thestop sequence. The recombination event is irreversible and thus the WT1 expressing cells and their descendants permanently express LacZ. Lox sites in the diagram are represented by green triangles (Diagram provided by Bettina Wilm).

embryos were decapitated. Embryos were dissected under a Nikon SMZ1000 zoom stereomicroscope (Nikon Instruments Inc, Melville, USA). Transverse incisions were made caudally and rostrally to the hind limb bud using the hypodermic needles. A sagittal section was then made through the spinal cord of the isolated hind limb region to separate the left and right sides. Finally, the limb was removed from the base of each side and the kidney rudiments were exposed. The hypodermic needles were use to tease the surrounding tissue away from the kidney rudiment, thus freeing it.

2.2.2 Neonatal and adult kidney

CD-1 mice (Charles River) were sacrificed with CO_2 and/or cervical dislocation. The mice were laid on their back and sprayed with 70% ethanol in order to reduce

the contamination with hair during the dissection. The skin was removed and the abdominal cavity was opened using surgical scissors (Sigma) and watchmaker forceps (Sigma). The internal organs were removed and the kidneys extracted.

2.3 Preparation of frozen mouse kidney sections

Whole kidneys were surgically removed from CD1 mice (Charles River) at different ages: 1 day old, 6 days old, 2 weeks old, three weeks old, and adult. At least three mice, from different litters, were analysed per time point. Mice were sacrificed with CO_2 and/or cervical dislocation.

2.3.1 Fixation and mounting of kidneys

The kidneys were fixed in 4% (w/v) paraformaldehyde (PFA) (Sigma, Poole, UK) in phosphate buffered saline (PBS) (see "Buffer recipes", page 82) for 1 hour at room temperature in the dark. In the case of adult kidneys, the kidneys were cut into 3 parts and left in PFA for 4 hours. The kidneys were then washed three times in PBS and mounted on a Peel-A-Way® embedding mould (Polysciences Europe GmbH, Eppelheim, Germany) using cryo-embedding solution (cryo-M-bed, Bright Instruments Company Ltd. Huntingdon, UK), and were then frozen in liquid nitrogen-chilled isopentane (Sigma). Transverse sections of the frozen kidneys were cut at 7 μm using a cryostat set at -20 °C (HM505N, Microm International, Walldorf, Germany) and C35 Feather microtome blades (VWR. Lutterworth, UK). The sections were placed on gelatine/$CrKSO_4$ subbed (see Preparation of subbed slides, page 58) twinfrost

glass microscope slides (76×26×0.8 mm) (VWR). Frozen sections were stored at -80 °C.

Seven day old WT1/Cre recombinase β-galactosidase transgenic mice kidneys were received in PBS already fixed in 4% PFA. The same protocol for embedding was followed as described above.

2.3.2 Preparation of subbed slides

Slides were soaked for 10–15 minutes in absolute ethanol (University of Liverpool solvents service) and washed five times in reverse osmosis water. The slides were then immersed for 25 seconds in a solution of 0.5% (w/v) gelatin from porcine skin type A (Sigma) and 0.05% (w/v) of $CrKSO_4$ (Sigma) in reverse osmosis water.

2.4 Immunostaining

2.4.1 Immunostaining of frozen sections

After being removed from the -80 °C freezer, the sections were left for 5 minutes at room temperature to defrost. The position of the sections on the slide was marked by circling them with a hydrophobic pen (DAKO, Copenhagen, Denmark) to delineate the area of tissue to be stained. The samples were blocked for one hour at room temperature with 0.1% Triton X-100 (Sigma) and 10% (v/v) serum (Sigma) in PBS. The serum used in the blocking, primary antibody, and secondary antibody solutions was goat, bovine, or chicken serum (Sigma) depending on the antibody used. When possible, the serum used for blocking was from the species in which the secondary antibody was raised.

After one hour the blocking solution was removed, and the sections were incubated with the primary antibody solution, which contained the primary antibody, or for dual staining two primary antibodies, in 0.1% (v/v) Triton X-100 and 1% (v/v) serum in PBS. The section was then left overnight at 4 °C in a humidified chamber. Details of the different primary antibodies used are given in Table 2-1. The next day the slides were washed three times in PBS at room temperature, before being incubated with the secondary antibody in 0.1% Triton X-100 and 1% (v/v) serum in PBS for two hours at room temperature in a humidified chamber in the dark. Details of the different secondary antibodies used are given in Table 2-2. The sections were then washed three times in PBS at room temperature. The blocking solution and the primary and secondary antibody solutions were centrifuged prior to use at 13400× *g* for 6 minutes in a Microcentaur centrifuge (Sanyo Electric Co., Ltd, Osaka, Japan).

Finally, to visualize the nuclei of the cells, the sections were incubated with DAPI (4′,6-diamidino-2-phenylindole, dihydrochloride) (Invitrogen, Paisley, UK) 1:100,000 in PBS for 3 minutes at room temperature. The sections were then washed three times in PBS at room temperature, and mounted in Dakocytomation fluorescent mounting medium (DAKO). Clear nail polish was then applied to seal the slides. The sections were observed under a Leica DM2500 fluorescence microscope (Leica, Heidelberg, Germany), and images were captured using a DFC350FX camera. Samples were also observed under LEICA AOBS SP2 confocal microscope.

Table 2-1 Primary antibodies used in this study.

Antibody	Host	Isotype	Concentration	Supplier
Pax-2	Rabbit	Polyclonal IgG	1:200	Covance
β-Galactosidase	Rabbit	Polyclonal IgG	1:5000	Cappel/ICN
β-Galactosidase	Chicken	Polyclonal IgG	1:750	Abcam
WT1 clone 6F-H2	Mouse	Monoclonal IgG1	1:500	Upstate (Millipore)
WT1	Rabbit	Polyclonal IgG	1:500	Sigma
pan Cytokeratin	Mouse	Monoclonal IgG1/IgG2a	1:500	Sigma
AQP1	Goat	Polyclonal IgG	1:200	Santa Cruz
AQP2	Goat	Polyclonal IgG	1:200	Santa Cruz
PECAM-1 (CD31)	Rat	Monoclonal IgG2a	1:100	BD Pharmingen
vWF	Rabbit	Polyclonal IgG	1:100	Sigma
αSMA	Mouse	Monoclonal IgG2a	1:500	Sigma
Synaptopodin	Mouse	Monoclonal IgG1	ready to use	Fitzgerald
Podocin	Rabbit	Polyclonal IgG	1:500	Alpha Diagnostic
Calbindin	Rabbit	Polyclonal IgG	1:200	Chemicon

Table 2-2 Secondary antibodies used in this study.

Antibody	Host	Isotype	Concentration	Supplier
anti-mouse AlexaFluor® 488	Goat	IgG1	1:500	Invitrogen
anti-mouse AlexaFluor® 488	Goat	IgG2a	1:500	Invitrogen
anti-mouse AlexaFluor® 594	Donkey	IgG	1:500	Invitrogen
anti-goat AlexaFluor® 488	Donkey	IgG	1:500	Invitrogen
anti-rabbit AlexaFluor® 594	Chicken	IgG	1:500	Invitrogen
anti-rabbit AlexaFluor® 594	Goat	IgG	1:500	Invitrogen
anti-chicken AlexaFluor® 488	Goat	IgG	1:400	Invitrogen
anti-rat AlexaFluor®488	Chicken	IgG	1:1000	Invitrogen

In every staining a negative control, in which the primary antibody was omitted, was included in order to ensure that non-specific binding of the secondary antibody was not occurring.

Wild-type mouse kidneys were used as controls for β-galactosidase immunostaining in experiments using transgenic mouse kidneys.

2.4.2 Immunostaining of cultured cells

Before the cells were stained, the culture medium was aspirated from the dishes and the cells were fixed for 10 minutes in 4% (w/v) PFA (Sigma) in PBS at room temperature and in the dark. After fixation the dishes were washed three times in PBS at room temperature and a ring was drawn with the hydrophobic pen (DAKO) around the area of cells. The cells were immunostained as described above.

2.4.3 Immunostaining of kidney rudiments

In order to stain kidney rudiments, the kidneys from E11.5 embryos were dissected and plated in a tissue culture petri dish (Sigma) for 48 hours in kidney rudiment culture medium. Then the rudiments were fixed for 20 minutes in 4% (w/v) PFA. The dishes were then washed three times in PBS at room temperature. The kidney rudiments were double stained for Pax-2 and WT1 as described above. The rudiments were then mounted in Dakocytomation fluorescent mounting medium (DAKO) and observed under LEICA AOBS SP2 confocal microscope.

2.5 Quantification of Pax-2 during kidney development

To quantify Pax-2 expression at different stages of kidney development kidneys were collected from mice at 3, 7, 14, and 21 days after birth. Three different kidneys were analysed per time point, and three different sections per kidney were studied. Kidneys were stained for Pax-2 (Covance) and nuclei were counterstained with DAPI. Images of the cortex-medulla and the papilla at 20× magnification were taken with a Leica DM2500 microscope using a DFC350FX camera.

Image J software (Rasband, 2008) was used to quantify the fluorescence intensity of Pax-2 and DAPI. Grayscale tiff images for each stain were thresholded at 30 to 255, and the number of selected pixels and signal intensities were measured (Figure 2-2). The ratio of total Pax-2 intensity to total DAPI intensity for each field of view was calculated. The mean Pax-2:DAPI ratios for the four time points were compared using an ANOVA, and a Tukey HSD *post hoc* test was applied to identify significantly different time points.

2.6 Lectin staining

The frozen sections were thawed at room temperature for 5 min. The kidney sections were co-stained with Pax-2 and a lectin. The sections were washed three times in Tris buffered saline (TBS) (see Buffer recipes, page 82), and incubated with the lectin at a concentration of 10 µg/ml (v/v) in TBS for 2 hours at room temperature. After the incubation time the lectins were washed three times in TBS.

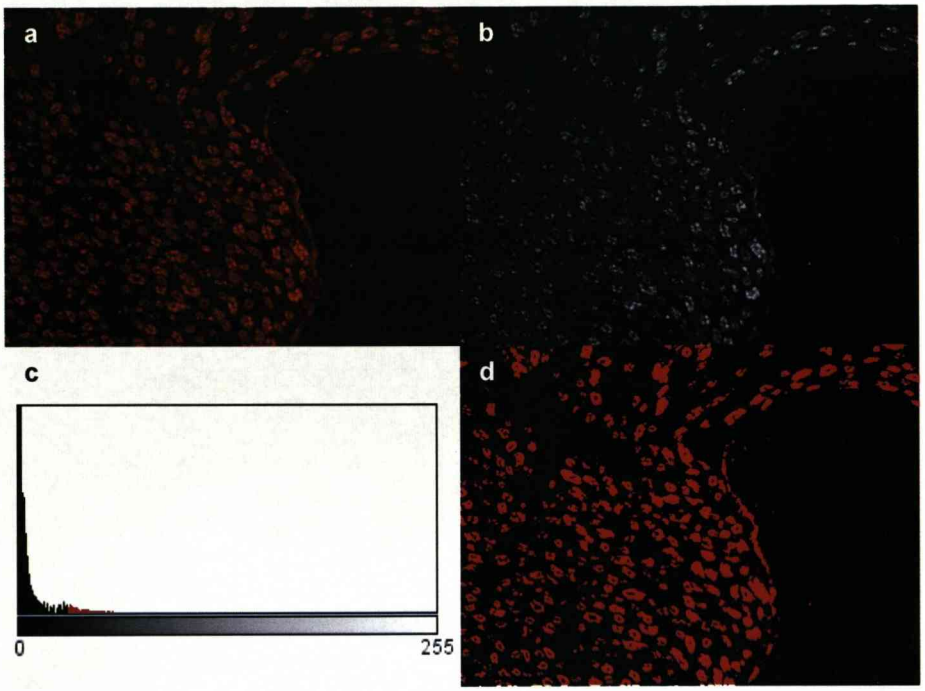


Figure 2-2 Pax-2 and DAPI levels in kidney sections were measured using Image J. (a) Micrograph showing immunostaining of a section of 3 day old mouse kidney for Pax-2 (red). (b) Grayscale equivalent of (a); the intensity of Pax-2 staining is represented by the whiteness of each pixel, which can be measured on a scale of 0 (black) to 255 (white). (c) Histogram of the intensity of all pixels in the micrograph. The red region represents the "thresholded" pixels, in this case those with a signal intensity of 30 or greater. The integral of this area gives a measure of the total level of signal from Pax-2 staining on the slide. (d) A visual representation of the thresholding applied in (c), all pixels selected using this thresholding are coloured red.

The sections were then blocked with 0.1% (v/v) Triton X (Sigma) and 10% (v/v) chicken serum (Sigma) in PBS for one hour at room temperature. After incubation time the blocking solution was aspirated and immunostained for Pax-2 and DAPI as described previously. Pax-2 was visualized with a secondary antibody conjugated with AlexaFluor® 594.

The lectins used were taken from the Fluorescein Lectin Kit I, the Fluorescein Lectin Kit II, and the Fluorescein Lectin Kit III (Vector Laboratories Ltd., Peterborough, UK). All the lectins were directly conjugated with fluorescein.

The lectins contained in the kits were concanavalin A (Con A), *Dolichos biflorus* Agglutinin (DBA), peanut agglutinin (PNA), *Ricinus communis* Agglutinin I (RCA120) , soybean agglutinin (SBA), *Ulex europaeus* Agglutinin I (UEA I), Wheat germ Agglutinin (WGA), *Griffonia (Bandeiraea) simplicifolia* Lectin I (GSL I), *Lens culinaris* Agglutinin (LCA), *Phaseolus vulgaris* Agglutinin (PHA-E), *Phaseolus vulgaris* Agglutinin (PHA-L), *Pisum sativum* Agglutinin (PSA), Succinylated WGA (sWGA), *Sophora japonica* Agglutinin (SJA), *Datura stramonium* Lectin (DSL), *Erythrina cristagalli* Lectin (ECL), *Griffonia (Bandeiraea) simplicifolia* Lectin II (GSL II), Jacalin, *Lycopersicon esculentum* (Tomato) Lectin (LEL), *Solanum tuberosum* (Potato) Lectin (STL), *Vicia villosa* Lectin (VVL).

2.7 Alkaline phosphatase staining

The medium was aspirated from the cells and they were washed once in Tris-HCl pH 9.2. Tris-HCl was aspirated and the alkaline phosphatase solution was added to the cells and incubated at room temperature for 5–20 min. The alkaline phosphatase staining solution was prepared by adding 2 mg of naphthol AS-MX phosphate (Sigma) and 10 mg of Fast Red TR (Sigma) to 10 ml Tris-HCl pH 9.2. The alkaline phosphatase staining solution was removed from the cells and they were then rinsed once in Tris-HCl.

2.8 Disaggregation of neonatal mouse kidneys

The following disaggregation protocol was based on those described by Cook *et al.* (1987) and Vielhauer *et al.* (2003).

The kidneys were surgically removed from 2–6 days old CD-1 mice immediately after killing the animals by CO_2 and/or cervical dislocation. The kidneys were placed in cold Paris Buffer (see Buffer recipes, page 82). The kidneys were cut into small pieces of <1 mm in ice-cold Paris Buffer, transferred to a 15 ml centrifuge tube (Falcon), and left for a few minutes for the tissue pieces to settle under gravity. The Paris buffer was removed and the kidneys were washed three times by re-suspending them in Hank's buffered saline solution (HBSS) with calcium and magnesium (Gibco), leaving them for a few minutes to settle under gravity, and then removing the supernatant.The kidneys were then immersed for 20 min at 37 °C in the enzymatic solution containing 1 mg/ml of collagenase type I (Sigma) and 0.1 mg/ml of deoxyribonuclease I (DNase I) (Sigma) in HBSS containing calcium and magnesium (Invitrogen).

The cell suspension was washed two times with HBSS containing calcium and magnesium at room temperature. Between washes the cells were left to settle for a few minutes under gravity.

The tissue was incubated in 0.5 mM EDTA (Sigma) for 20 minutes at 37 °C. The supernatant, containing isolated individual cells, was then removed and kept on ice.

The remaining pellet was incubated with 5 ml of 1 mg/ml collagenase I in HBSS containing calcium and magnesium for 20 minutes at 37°C.

After the incubation, the whole suspension was passed twice through a 21-gauge needle and then twice through a 23-gauge hypodermic needle, and left to settle on ice for 5–10 min. Both supernatants were pooled and centrifuged for 2.5 minutes

at 200× *g* and washed twice in HBSS (containing calcium and magnesium) at room temperature, after which, the pellet was re-suspended in 1 ml of the HBSS.

The cell suspension was then poured gently into a 15 ml falcon tube containing 7 ml of HBSS containing calcium and magnesium, so that the cell clumps settled at the bottom of the tube, whilst individual cells remained in suspension. After 10 minutes the supernatant containing individual cells was collected and the pellet discarded.

The whole cell suspension was passed through a 30 μm pre-separation filter, centrifuged for 2.5 min at 200× *g* and re-suspended in MACS® separation buffer (10% BSA) (Miltenyi biotec) for MACS isolation of the cells, or in normal culture medium if the cells were to be plated without MACS separation.

2.9 Isolation of cells using magnetic cell sorting (MACS®)

Six kidneys were disaggregated as described above to obtain a total of 2.5×10^6 cells. Cells were counted in a Neubauer haemocytometer (Hausser Scientific, Horsham PA, USA). Disaggregated kidney cells were divided into three equal fractions. One fraction was used as a control, and no isolation procedure was performed on it. The second fraction was incubated with magnetic beads coated with PNA lectin (LodeStars™ 2.7 Carboxyl. Polymer laboratories) for 1.5 h at 37 °C under agitation, and the third fraction was incubated with uncoated beads under the same conditions.

Cells incubated with magnetic beads were passed through a MS column (Miltenyi Biotec) that was placed in a magnetic field (MiniMACS™ separator, Miltenyi

Biotec). The column was then washed through with MACS separation buffer and the washed fraction, containing the cells that were not retained by the magnetic field, was collected; hereafter referred to as the unselected fraction. The cells bound to the magnetic beads (PNA-coated and control) were retained in the column.

The magnetic field was then removed, and the fraction of cells retained by the magnetic field was collected by washing the column with MACS separation buffer; hereafter referred to as the selected fraction. The selected fraction was seeded onto 3.5 cm tissue culture dishes coated with a monolayer of STO feeder cells and Advanced DMEM serum free medium. Two dishes were cultured per fraction. One dish was fixed in 4% (w/v) PFA after 24 h, and the other was expanded in culture for further analysis. This experiment was performed three times.

The dishes fixed 24 h after plating were stained for Pax-2 and PNA lectin. Ten different fields of view at 20× magnification were analysed per fraction. The total number of cells was established by counting the number of cells stained with DAPI, and the number of cells positive for Pax-2 was also counted. The percentage of Pax-2 positive cells was then established.

Cells were analysed after two weeks in culture. They were fixed in 4% PFA and stained for Pax-2. The number of colonies on each dish was counted, and the colonies were classified as Pax-2 positive or Pax-2 negative. When more than 50% of the cells in a colony were positive the colony was considered Pax-2

positive, and when less than 50% of the cells in the colony were negative, the colony was considered negative.

2.9.1 Removal of magnetic beads

The following disaggregation protocol was based on the protocol described by Gomez *et al.* (1998). After MACS, the fractions of cells selected with PNA were resuspended in 1 ml of Hank's buffer saline solution (HBSS) containing 5% fetal calf serum (FCS) and 200 mM D-galactose (Vector laboratories), and incubated for 10 min at room temperature with rotation. The suspension was passed through the columns placed in the magnetic field, and was then washed 5 times with HBSS with 5% FCS. The cells were centrifuged at 200× *g* for 5 min and resuspended in the adequate growth media.

2.10 Cell culture

All the cells used in this study were kept in a cell incubator at 37 °C and 5% CO_2 (Sanyo). Along the study, the cells were grown on different substrates with different culture media. All the protocols for coating the culture dishes and media recipes are listed below; however, details on how particular cells were cultured and the conditions used are explained in the relevant chapters.

2.10.1 Routine subculture

During this study all the cells were subcultured using the following protocol. The cell culture medium was removed and the cells were washed two times in PBS without calcium and magnesium (Invitrogen) that had been pre-warmed to 37 °C. The cells were detached from the dishes using a mixture of trypsin and EDTA

(Sigma) in PBS without calcium and magnesium to a final concentration of 0.05% trypsin/0.02% EDTA. The cells were incubated in the trypsin/EDTA for approximately 5 min at 37 °C, and gently shaken to detach the cells. Then the trypsin/EDTA was neutralized by adding Dulbecco's modified Eagle medium (D-MEM) (Invitrogen) supplemented with 10% (v/v) FCS (PAA. Pasching, Austria). The suspension containing the cells was then centrifuged at 200× *g* for 2.5 min. The supernatant was aspirated, and the pellet containing the cells was resuspended in the growth medium.

2.10.2 Preparation of culture substrates

Preparation of Matrigel coated dishes

Matrigel® (Becton Dickinson, Oxford, UK) was thawed at 4 °C overnight on ice. Glass and plastic pipettes, pipette tips, tissue culture plates and microcentrifuge tubes (Eppendorf, Hamburg, Germany) were kept at -20 C until they were needed. The Matrigel® stock was diluted 1:2 (v/v) in cold Knockout™ DMEM (Invitrogen) and stored in aliquots at -20 °C.

To prepare Matrigel® coated plates, the aliquots were thawed slowly at 4 °C and diluted at 1:15 (v/v) in cold Knockout™ DMEM (Invitrogen) (for a final dilution of 1:30). The diluted Matrigel® was added to 3.5 cm cell culture dishes (Nunc, Rochester, NY). The dishes containing Matrigel® were kept at 4 °C for up to a week before they were used. The Matrigel® solution was removed immediately before use.

Preparation of STO feeder cell layers

Gelatinisation of cell culture dishes

Cell culture dishes (Nunc) were coated with 0.1 % (w/v) porcine gelatin solution (Sigma) in distilled water for 30 min at room temperature.

Routine subculture of STO cells

The STO (Sandoz inbred mouse embryo-derived thioguanine and ouabain-resistant) fibroblast cell line was a gift from Neil Smyth (University of Southampton, UK). STO cells were thawed from frozen stocks and cultured in DMEM with 10% added serum (see Culture media, page 78) in 10 cm cell culture dishes (Corning. NY, USA).

After 3 days, when the cells were confluent, the cells were detached from the tissue culture dish using trypsin/EDTA as described above. After trypsinization the cells were resuspended in DMEM with 10% added serum and re-plated onto fresh gelatinized culture dishes (Corning).

Preparation of STO feeder cell layers

When STO cells were confluent, they were treated with 20 μg/ml of mitomycin-C (Sigma) in DMEM with 10% added serum, and incubated at 37 °C in a humidified incubator in an atmosphere of 5% CO_2 for 2.5 h. After the incubation time, the mitomycin-C was aspirated and the cells were washed two times in 10 ml PBS (calcium and magnesium free) pre-warmed to 37 °C. The cells were then trypsinized, as previously described, and split into 3.5 cm gelatinized tissue culture dishes (Nunc). Every 10 cm dish with confluent STO cells was split into sixteen 3.5 cm STO feeder cell dishes.

Sodium deoxycholate treated STO feeders

Culture medium was removed from STO feeder dishes and the cells were washed three times in warm PBS (Invitrogen). The PBS was aspirated and a solution containing 0.5% sodium deoxycholate and 1 mM phenylmethyl sulfonylfluoride in 10 mM Tris-Cl buffered saline at pH 8.0 was added to the cells. The cells were incubated in this solution for thirty minutes at 4 °C on a rocking platform (Hedman *et al.*, 1979). The deoxycholate solution was removed and the dishes were washed 5 times in PBS. The cells were kept at 4 °C until use.

Fibronectin coated dishes

Cell culture dishes (Nunc) were coated with a 50 µg/ml solution of fibronectin (Chemicon) in PBS without calcium and magnesium, and incubated for 1 h at 37 °C. The fibronectin solution was aspirated immediately before plating the cells.

2.11 Differentiation of kidney stem cells

2.11.1 In vitro differentiation of KSC

Putative kidney stem cells (pKSC) (see Chapter 6, Characterisation and differentiation of putative KSC) were plated onto uncoated plastic culture dishes (Nunc). The media used was DMEM with 10 % added serum (see Culture media, page 78). The media was changed every two days and the cells were subcultured when confluent.

2.11.2 Extrarenal differentiation: Adipogenesis

For adipogenic differentiation, pKSC and differentiated putative kidney stem cells (dpKSC) were incubated for 2–3 weeks in adipogenic medium (see Adipogenic medium, page 81). The cells were fixed as described above and were stained for 20 min with 0.5% (w/v) oil red O (Sigma) in isopropanol (Sigma). The cells were washed three times in distilled water to remove excess dye, mounted in 80% (v/v) glycerol in water (Sigma) and were observed under a Leica DM2500 microscope (Leica). Images were captured using a DFC350FX camera.

2.12 Integration of kidney stem cells into developing kidney rudiments

A pregnant CD-1 mouse (Charles River) was sacrificed at 11.5 days following timed mating by cervical dislocation, and the embryos were isolated. Twelve kidney rudiments were dissected from E11.5 mouse embryos and placed in a 1.5 ml microcentrifuge tube containing 100 µl of kidney rudiment culture medium (see Kidney rudiment culture medium, page 81).

Twelve kidney rudiments were dissected from 11.5 day mouse embryos as described above. Kidney rudiments were transferred into a 3.5 cm dishes containing 2× trypsin/EDTA (Sigma), and incubated for five minutes at 37 °C and 5% CO_2. After the trypsin incubation step, the kidneys were transferred to a fresh dish containing the kidney rudiment culture medium, and left for 15 minutes at 37 °C to inhibit the trypsin.

The kidneys were transferred to a fresh 1.5 ml microcentrifuge tube containing 100 µl of kidney rudiment culture medium, and were dissociated by gentle pipetting in order to disrupt cell aggregates.

Putative kidney stem cells (pKSC) were labelled with Vybrant® carboxyfluorescein diacetate, succinimidyl ester cell tracer kit (Invitrogen). A 3.5 cm confluent culture dish containing pKSC was trypsinized as described before (see routine subculture section) and incubated in 1 ml of cell tracker staining solution (0.5 µM of cell tracker in Advanced DMEM medium). The cells were incubated at 37 °C 5% CO_2 for 45 minutes. The cells were then washed three times by adding 3 ml of Advanced DMEM medium at room temperature and centrifuging at 1400× *g* for 3 minutes. The cells were resuspended in 1 ml of Advanced DMEM and they were counted using a haemocytometer (Hausser Scientific).

The 100 µl solution containing disaggregated cells was mixed by gentle pipetting with 100 µl of Vybrant®-labelled kidney stem cells in a ratio of 2:1 (two parts kidney cells to one part labelled KSC). The cell suspension was then centrifuged at 1,400× *g* for 3 min in order to obtain a compact pellet.

The pellet was then collected with a pipette and placed on a 1.2 µm isopore membrane filter (Millipore; Sigma). The filter was then placed on a stainless steel metal grid inside a culture dish containing the culture medium which reached the sample by capillary (Figure 2-3).

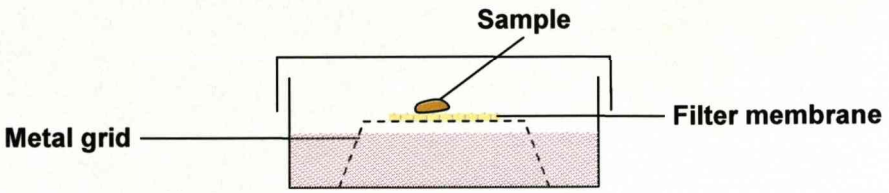


Figure 2-3 Diagram showing transfilter culture of kidney aggregate.

The cells were cultured for 24 h in kidney rudiment culture medium with 20 μM of the Rho-kinase inhibitor Y27632 (Calbiochem). The media was then replaced with kidney rudiment culture medium without Y27632. The kidney rudiment was fixed after 4 days in 4 % (w/v) PFA for 45 minutes in the dark at room temperature. After fixation the kidney rudiments were washed three times in PBS and stained for Calbindin as described in the immunostaining section (page 58).

This protocol to integrate kidney stem cells into developing kidney rudiments was kindly provided by Mathieu Unbekandt from the University of Edinburgh.

2.13 Cell growth curves

In order to compare the growth rates of pKSC and dpKSC the proliferation rates were measured and growth curves were established. The cells in both conditions were subcultured when they were confluent, approximately every 2–3 days, and the number of cells counted over seven passages. Three different replicates were performed per condition. A linear regression analysis was performed to determine the rate of proliferation as a function of time. From the results of the regression the time required for a doubling of the population was calculated for each group.

2.14 Clonogenic assay

In the first attempt cells were trypsinized and single cells were pipetted with a mouth pipette (Sigma) and plated in a 96 well plate (Nunc). The experiment was repeated twice. The cells were cultured in STO conditioned medium 2.5% FCS. In the second experiment the cells were trypsinised, and were resuspended in DMEM with 10% FCS at a final density of 1 cell per 50 µl. Cells were plated in 96 well plates (Corning® Costar® Sigma) at 50 µl per well, in order obtain one cell per well. All the wells were examined, and wells containing no cells or more than one cell were discarded. Three replicates of this experiment were performed.

2.15 RT-PCR

2.15.1 RNA extraction

To isolate RNA from cultured cells, first the cell medium was removed from the culture dishes containing the isolated kidney cells, and 1 ml of TRIzol® Reagant (Invitrogen) was added. The TRIzol was left for a few minutes, and then the cells were lysed by pipetting. The TRIzol/cell solution was then transferred to a 1.5 ml microcentrifuge tube.

When embryonic kidney rudiments were collected for RNA extraction, five kidney rudiments were place in a microcentrifuge tube containing 1 ml of TRIzol, and they were homogenized using a pellet pestle motor and a polypropylene pestle (Sigma).

RNA extraction for both cells and kidney rudiments then proceeded as follows. 200 µl of chloroform were added, and the samples were mixed by inversion for

15 seconds. The samples were then centrifuged at 4 °C for 15 minutes at 12,000× *g*, which separated the mixture into three distinct phases.

The upper aqueous phase, containing RNA, was transferred to a fresh microcentrifuge tube containing 1 µl of glycogen (1 µg/µl) (Boehringer Mannheim GmbH, Mannheim, Germany) and 0.5 ml of isopropanol (Sigma). The contents of the tube were then mixed by inverting five or six times. The samples were incubated overnight at -20 °C.

The chilled samples were centrifuged at 4 °C for 10 minutes at 12,000× *g*. The supernatant was discarded and 1 ml of 75% ethanol (diluting ethanol absolute for molecular biology (Sigma) in nuclease-free water (Sigma) was added to wash the pellet. The pellet was washed by vortexing, and was then settled by centrifugation at 4 °C for 5 min at 4,400× *g*. The ethanol was removed from the RNA samples by pipetting, and the pellets were left to air dry for a few minutes. The RNA pellets were finally dissolved in 15 µl of nuclease-free water (Sigma).

2.15.2 DNase treatment

For DNAse treatment 8 µl of RNA solution was incubated with 1 µl of RQ1 DNase buffer (Promega, Madison, WI, USA) and 1 µl of DNase (Promega) for 30 min at 37 °C. After the incubation, 1 µl of Stop Buffer (Promega) was added and the solution was incubated for a further 15 minutes at 60 °C.

The concentration and quality of the extracted RNA were measured using a NanoDrop™ 1000 Spectrophotometer (NanoDrop Technologies, Wilmington, USA). RNA quality was assessed using the 280/260 nm and 280/230 nm

absorbance ratios. The former ratio provides an assessment of RNA integrity, while the latter provides an indication of contamination of the sample with salts or organic compounds. Sample with either ratio less than 1.8 were discarded.

2.15.3 cDNA synthesis

For cDNA synthesis, 5 µl of nuclease-free water and 2 µl of a 100 ng/µl stock solution of random hexamers (Abgene, Portsmouth, USA) were added to 5 µl of DNase treated RNA, and the samples were incubated at 80 °C for 3 min. The samples were chilled briefly on ice and the tubes were pulse centrifuged to collect the contents. In the next step, 4 µl 5× 1st strand buffer (Invitrogen), 2 µl of 100 mM DTT (dithiothreitol) (Invitrogen), and 1 µl 10 mM dNTP mix (Bioline Ltd, London, UK) were added to the sample. The reaction mixture was mixed gently, and then incubated at 42 °C for 2 min. After 2 minutes, 1 µl of Superscript III^{TM} reverse transcriptase (Invitrogen) was added. The samples were then incubated for 60 minutes at 50 °C, followed by a final incubation step at 70 °C for 15 min to inactivate the enzyme. The samples were stored at -20 °C until they were used.

2.15.4 Oligonucleotide primers

All primers were obtained from Sigma. The primers were resuspended in 1× TE buffer (Cambrex, Rutherford, NJ) to a final concentration of 100 µM. Working dilutions of 5 µM were prepared in nuclease-free water (Sigma) and kept at -20 °C until they were needed. All the primers were used at 0.2 µM.

2.15.5 PCR

The 20 µl PCR reaction consisted of 2 µl of 10× NH_4 Buffer (Mg^{2+} free), 0.5 µl of $MgCl_2$ Solution (50 mM), 0.5 µl dNTP mix (10 mM stock), 0.5 µl Taq DNA polymerase (all from Bioline), 0.8 µl of the forward and reverse primers (0.5 µM), 14.4 µl of nuclease-free water (Sigma), and 0.5 µl of cDNA (200 ng/µl).

The cycling conditions were as followed: An initial step of 3 minutes at 94 °C, followed by 38 cycles of 94 °C for 30 seconds, 62 °C for 30 seconds and 72 °C for 45 seconds, followed by a final extension of 72 °C for 10 min.

The PCR products were detected by gel electrophoresis on a 1.5% (w/v) agarose gel (Bioline) in 1× TAE, with ethidium bromide solution (Sigma) added to the gel at the concentration 0.5 µg/ml. 2 µl of 6× loading buffer (see Buffer recipes, page 82) was added per 5 µl of sample, and the samples were run for 30 minutes at 100 V. The gel was visualized with a UV transilluminator (ChemiImager 4400, Alpha Innotech Corporation, San Leandro, CA, USA).

In all PCR reactions Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as reference gene.

2.16 Culture media

DMEM with 10% added fetal calf serum

- High glucose Dulbecco's Modified Eagle medium (DMEM) (without sodium pyruvate, with 4,500 mg/l Glucose) (Invitrogen)
- 10% FCS (PAA)

Table 2-3 Table showing the primers used in this study. The specificity of primers designed in-house was determined by sequencing of PCR products (University of Dundee sequencing service). F – forward primer; R – reverse primer.

Primer	Sequence	Product size	Source
Pax-2	F: GCGTCCTTCCTATCCCGATG R: TTGCCTGAGAACTCGCTCCC	303 bp	in-house
WT1	F: CCAGTGTAAAACTTGTCAGCGA R: TGGGATGCTGGACTGTCT	234 bp	(Yamamoto *et al.*, 2006)
GDNF	F: TGCCAGCCCAGAGAATTCCA R: AGCCTTCTACTCCGAGACAG	176 bp	in-house
Sall1	F: GCACATGGGAGGCCAGATCC R: GGAAGCGTCCGCTGACTTGG	141 bp	in-house
Podocalyxin	F: CACGGGAGTCCCATTAGAGA R: TGACATGGAAGCCCACTACA	179 bp	(Kramer *et al.*, 2006)
Synaptopodin	F: GCCAGGGACCAGCCAGATA R: AGGAGCCCAGGCCTTCTCT	73 bp	(Orth *et al.*, 2005)
ZO-1	F: CTCATAGTTCAACACAGCCT R: TCATCTTCATCTTCTTCCACAG	148 bp	(Osafune *et al.*, 2006)
Desmin	F: CTCAAGGCCAAACTACAGGA R: CAGAATCGAATCCCTCAACG	133 bp	in-house
Megalin	F: CCTTGCCAAACCCTCTGAAAAT R: CACAAGGTTTGCGGTGTCTTTA	562 bp	(Erranz *et al.*, 2004)
CD2AP	F: TGAGGTAGGGCCAGTCAAAG R: CGAGTTGGGGAAATCATCAG	504 bp	(Kramer *et al.*, 2006)
THP	F: CTAACCCATCCTTGCCTTT R: TCACACACCAGCCCATCAC	250 bp	(Kramer *et al.*, 2006)
Ksp-cadherin	F: GCCGTGGCTACTTTACCTCTCTGTTA R: TGATAGGACAATGTGGCCTTCATCTC	165 bp	(Kramer *et al.*, 2006)
Neph1	F: GTGGCCTGGCATGTAAAAAT R: CTAGACATTGGCCGCGTATT	167 bp	(Kramer *et al.*, 2006)
Vimentin	F: TCTTGACCTTGAACGGAAAGTG R: CACATCGATCTGGACATGCT	123 bp	in-house
Cytokeratin8	F: AGGAGCTTATGAACGTCAAG R: CCCATAGGATGAACTCAGTC	161 bp	(Osafune *et al.*, 2006)
VEGF	F: GAGATAGAGTACATCTTCAAGCC R: TTTCTTTGGTCTGCATTCAC	201 bp	in-house
GAPDH	F: TGAAGCAGGCATCTGAGGG R: CTTTGGTCCGGATG ATCCTG	110 bp	in-house

- 2 mM L-glutamine (Invitrogen)
- 1:100 (v/v) 100× non-essential amino acids (Invitrogen)

Advanced DMEM

- Advanced DMEM (Invitrogen)
- 2 mM L-glutamine (Invitrogen)
- 5 μM β-mercaptoethanol (Invitrogen)
- 1:100 (v/v) penicillin/streptomycin (Gibco) was added to the medium during the first 2–3 weeks after kidney isolation to prevent infections and omitted later on.

Advanced DMEM with growth factors

- Advanced DMEM (Invitrogen)
- 2 mM L-glutamine (Invitrogen)
- 5 μM β-mercaptoethanol (Invitrogen)
- 100 ng/ml of FGF-2 (Sigma)
- 50 ng/ml and BMP-7 (Abnova, Taiwan)

STO-conditioned medium with 2.5% FCS

STO-conditioned medium was Advanced DMEM in which STO cells had been grown for two days, according to the following protocol.

When STO cells became confluent DMEM with 10% FCS medium was removed from the cells and replaced with Advanced DMEM. The STO cells were gown in Advance DMEM for a further two days and then treated with mitomycin C. The

STO feeder cells were then subcultured and grown in Advanced DMEM. The conditioned media was collected after two days and replaced with fresh Advanced DMEM. After two more days the conditioned media was collected and the STO feeder cells were discarded. Collected media was either stored at 4 °C for immediate use or at -20 °C for up to 2 weeks.

Prior to use STO conditioned media was centrifuged at high speed, and only the supernatant was collected in order to exclude unwanted STO cells and debris. Conditioned medium was then mixed in a 1:1 ratio with fresh Advanced DMEM contained 5% FCS.

Kidney rudiment culture medium

- Minimum Essential Medium (MEM) (Invitrogen)
- 2 mM L-glutamine (Invitrogen)
- 1:100 (v/v) penicillin/streptomycin (Invitrogen)

Adipogenic medium

From Peister *et al.* (2004):

- DMEM (Invitrogen)
- 10 % FCS
- 1×10^{6} M dexamethasone (Sigma)
- 5 μg/ml insulin (Sigma)
- 50 μM indomethacin (Sigma)
- 0.5 μM 3-isobutyl-1-methylxanthine (IBMX) (Sigma)

2.17 Buffer recipes

2.17.1 Paris buffer

From Cook *et al.* (1987):

- 20 mM Tris/HCl (Sigma)
- 125 mM NaCl (Sigma)
- 10 mM KCl (Sigma)
- 10 mM sodium acetate (Sigma)
- 5 mM glucose (Sigma)

2.17.2 Phosphate buffered saline (PBS)

From Hogan *et al.* (1994):

- 0.137 M NaCl (Sigma)
- 2 mM KCl (Sigma)
- 6 mM, Na_2HPO_4 (Sigma)

pH adjusted to 7.4–7.6

2.17.3 Tris buffered saline (TBS) 10×

From IHC world (IHC world, 2007):

- 0.5 M Tris base (Sigma)
- 9% NaCl (Sigma)

pH adjusted to 7.6

2.17.4 Tris-acetate-EDTA (TAE) 50×

- 242 g Tris base (Sigma)
- 57.1 ml glacial acetic acid (Sigma)
- 100 ml 0.5 M EDTA (Sigma)

pH adjusted to 8.0

2.17.5 Gel-loading buffer 6×

From CSH protocols (Cold Spring Harbor laboratory, 2006):

- 3 ml glycerol (Sigma)
- 25 mg bromophenol blue (Sigma)
- 10 ml of distilled H_2O (Sigma)

Chapter 3: The location, prevalence and origin of Pax-2 positive cells in the postnatal mouse kidney

3.1 Introduction

Several approaches have been attempted, during the past few years, to identify and isolate renal stem cells in humans and rodents. These have included isolation of low cycling cells (Maeshima *et al.*, 2003; Maeshima *et al.*, 2006; Oliver *et al.*, 2004), the identification of side populations using FACS (Challen *et al.*, 2006; Hishikawa *et al.*, 2005; Iwatani *et al.*, 2004), the use of specific culture conditions that favoured the proliferation of stem cells over differentiated cells (Gupta *et al.*, 2006; Kitamura *et al.*, 2005), and the use of specific stem cell markers (Bussolati *et al.*, 2005; Dekel *et al.*, 2006; Sagrinati *et al.*, 2006). Cells isolated using these techniques have presented various characteristics indicative of stem cells, such as clonogenicity and the expression of markers characteristic of undifferentiated cells (Bussolati *et al.*, 2005; Challen *et al.*, 2006; Dekel *et al.*, 2006; Gupta *et al.*, 2006; Hishikawa *et al.*, 2005; Kitamura *et al.*, 2005; Maeshima *et al.*, 2006; Oliver *et al.*, 2004; Sagrinati *et al.*, 2006). Furthermore, they have been shown to be capable of differentiation to a variety of specialised cell types (Bussolati *et al.*, 2005; Challen *et al.*, 2006; Dekel *et al.*, 2006; Gupta *et al.*, 2006; Kitamura *et al.*, 2005; Maeshima *et al.*, 2006; Oliver *et al.*, 2004; Sagrinati *et al.*, 2006), and integration into developing kidney structures (Maeshima *et al.*, 2006). They have also been indicated as having a role in renal regeneration following acute injury (Challen *et al.*, 2006; Dekel *et al.*, 2006; Hishikawa *et al.*, 2005; Kitamura *et al.*, 2005; Oliver *et al.*, 2004; Sagrinati *et al.*, 2006).

The cell surface antigen CD133 has been successfully used to isolate progenitor cells from human tissue (Florek *et al.*, 2005; Yin *et al.*, 1997). CD133, also known as prominin-1, is a glycoprotein containing five transmembrane domains with an extracellular N-terminus and a cytoplasmic C-residue. It has a molecular weight of 120 kDa in humans and 115 kDa in mice (Shmelkov *et al.*, 2005; Weigmann *et al.*, 1997).

CD133 polyclonal antibody detects prominin-1 in epithelial cells; in the case of the kidney this includes the brush border of the proximal tubular cells both in humans and mice (Florek *et al.*, 2005). The monoclonal antibody AC133 binds a glycosylation-dependent epitope of CD133. The AC133 antigen is down-regulated when cell differentiation occurs, and it is therefore this antibody that has been identified as being specific to human progenitor cells (Florek *et al.*, 2005; Yin *et al.*, 1997). The marker is a reliable indicator of progenitor cells as its expression is generally restricted to stem cells and progenitor populations, such as hematopoietic stem cells (Miraglia *et al.*, 1997; Yin *et al.*, 1997), endothelial progenitor cells (Peichev *et al.*, 2000), human central nervous system stem cells (Uchida *et al.*, 2000), and human embryonic epithelia (Corbeil *et al.*, 2000; Weigmann *et al.*, 1997). However, the monoclonal antibody AC133 was raised in mice (Bussolati *et al.*, 2005; Cai *et al.*, 2005; Corbeil *et al.*, 2000; Yin *et al.*, 1997), and does not react with mouse protein (Prof. Giovanni Camussi, University of Turin, pers. comm.).

Bussolati *et al.* (2005) showed that a population of CD133 expressing cells is resident in adult human kidney, and that these cells, when isolated, demonstrated properties that suggested that they may be renal stem cells.

Human and mouse prominin-1 are highly related proteins that share approximately 60% amino acid-sequence identity (Corbeil *et al.*, 1998), and they are both widely expressed in embryonic and adult tissue (Weigmann *et al.*, 1997; Yin *et al.*, 1997).

Cells expressing the marker CD133 have been successfully isolated from adult human kidney, and have furthermore been shown to possess stem cell like properties (Bussolati *et al.*, 2005; Sagrinati *et al.*, 2006). The cells identified by Bussolati *et al.* (2005) using CD133 were found in the interstitium of the renal cortex and co-expressed the transcription factor Pax-2. The lack of expression of hematopoietic markers CD34 and CD45 excluded the possibility that they originated from circulating hematopoietic stem cells that had become resident in the kidney and further supported the inference that the expression of Pax-2 suggested a renal origin. Pax-2 is essential in normal kidney development and is expressed in a similar pattern during kidney development in mice and humans (Dressler *et al.*, 1990; Eccles *et al.*, 1992; Ryan *et al.*, 1995).

All of these findings suggest the possibility of the existence of a progenitor population in mice similar to that previously described in humans. However, while these cells would be expected to express Pax-2, they would not express the AC133 epitope recognised by the CD133 monocolonal antibody. Pax-2 therefore

is more likely than CD133 to be an amenable marker for putative kidney stem cells in murine kidney.

Both metanephric mesenchyme and ureteric bud express Pax-2 in the early stages of kidney development (Ryan *et al.*, 1995). The ureteric bud is responsible for the formation of the collecting ducts, and only the cells from metanephric mesenchyme form the nephrons (Saxen, 1987). Therefore it would be necessary to determine the origin of Pax-2 positive cells found in postnatal mouse kidney to ensure they have nephrogenic potential. While both cell populations express Pax-2, only metanephric mesenchyme expresses the transcription factor WT1 (Figure 3-1) (Vize *et al.*, 2003).

To determine the origin of Pax-2 cells, WT1-Cre/Rosa26R mice were used (supplied by Dr Bettina Wilm, University of Liverpool). WT1-Cre is a transgenic line with Cre recombinase under the control of the WT1 promoter. Rosa26R is a transgenic line in which Cre-mediated recombination activates expression of the β-galactosidase reporter gene. When cells in WT1-Cre/Rosa26R mice express WT1, they also express Cre recombinase, which activates β-galactosidase, this recombination event is irreversible. In these transgenic mice WT1 expressing cells and their progeny will permanently express β-galactosidase (Figure 2-1).

In conclusion, it would be very important to determine whether a population of Pax-2 positive cells, as found in humans, is also present in mice. Furthermore, if such a population is present, it would also be necessary to establish the embryonic origin of the Pax-2 positive cells present in postnatal mouse. If these Pax-2 cells had a ureteric bud origin it would mean that they would be unlikely to

have any nephrogenic potential, in contrast, proving a metanephric mesenchymal origin would indicate that these cells could potentially form nephrons.

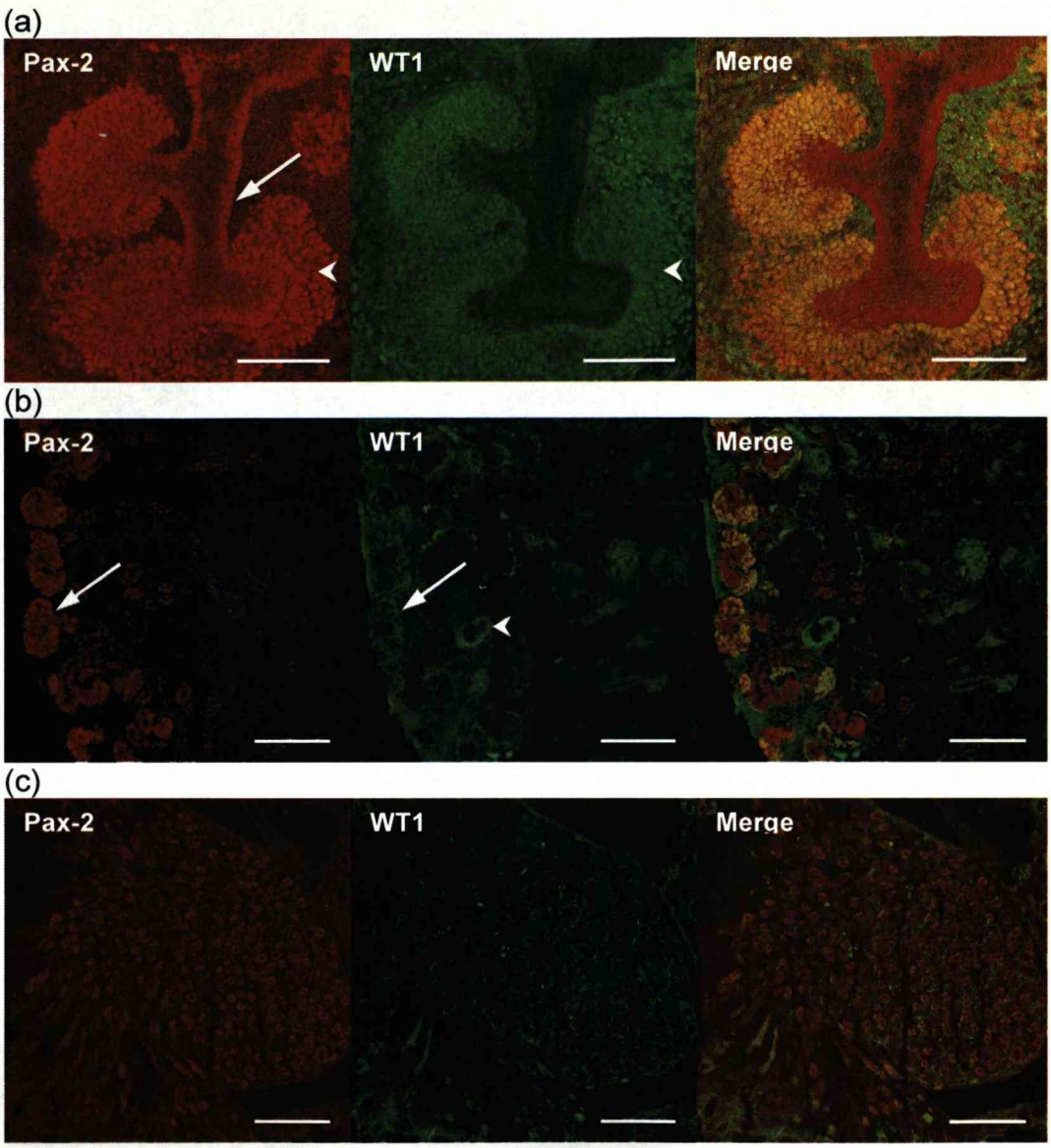


Figure 3-1 Fluorescence micrographs showing immunostaining of cultured kidney rudiments and 1 day old mouse kidney for Pax-2 and WT1. (a) Kidney rudiments of E11.5 embryo cultured for two days showing Pax-2 expression in ureteric bud (arrow) and metanephric mesenchyme (arrowhead) and WT1 expression in metanephric mesenchyme (arrowhead). (b) Cortex of 1 day old mouse kidney showing Pax-2 positive cells in the nephrogenic area (arrow) and WT1 in some cells of the nephrogenic area (arrow) and developing glomerular epithelium (arrowhead). (c) Papilla of 1 day old mouse showing Pax-2 positive cells and absence of WT1 expression. Scale bars 100μm.

3.1.1 Aims

In order to investigate if postnatal mouse kidney contains a population of Pax-2 positive cells equivalent to the Pax-2 population found in human kidney, immunostaining for Pax-2 was performed at different stages of kidney development.

To trace the lineage derivatives of metanephric mesenchyme and determine where Pax-2 positive cells originate, transgenic mice were used to permanently label WT1 expressing metanephric mesenchyme cells and their descendants.

3.2 Results

3.2.1 The location of Pax-2 positive cells in the postnatal mouse kidney

Pax-2 expression in day 1 mouse kidney

In neonatal mice at day 1 (d1), the expression of Pax-2 appeared very similar to the expression observed in late embryonic kidney by Dressler *et al.* (1992). Pax-2 was found to be expressed in the nephrogenic area in the outer cortex, specifically, in the condensed mesenchyme surrounding the ureteric bud, in the comma-shaped bodies, the S-shaped bodies, and in the tips of the ureteric bud (Figure 3-2). Pax-2 positive cells were also present in some of the tubular structures in the cortex and outer and inner medulla. A high number of Pax-2 positive cells were found in the renal papilla at this stage. Some cells appeared to express higher levels of Pax-2 than others. Most of the Pax-2 positive cells in the papilla were located in tubular structures although a few of them appeared to be

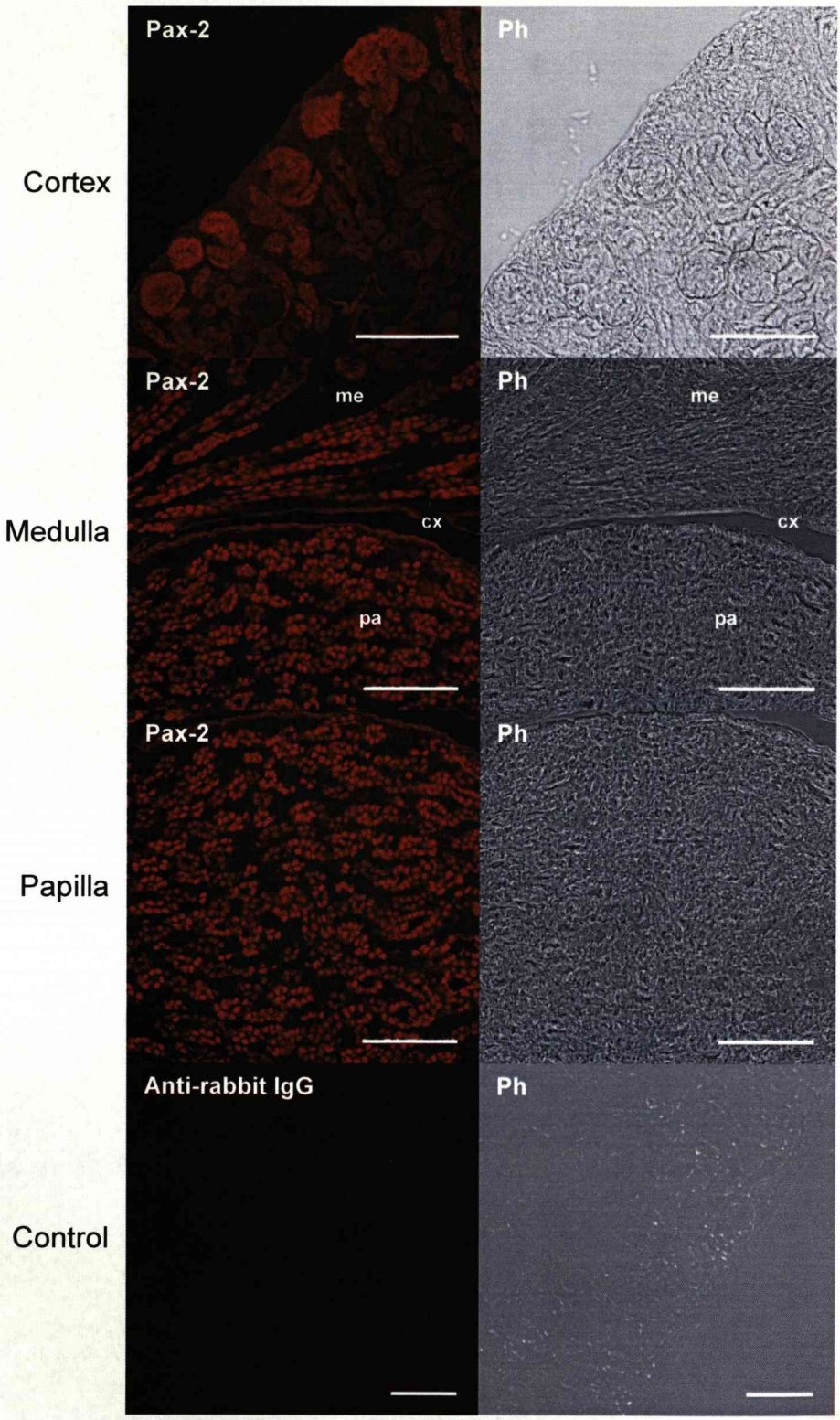


Figure 3-2 Confocal micrographs showing Pax-2 expression in frozen sections of 1 day old neonatal mouse kidney. An image of the cortex from a control staining in which the primary antibody was omitted is also shown. The images are representative of five individual kidneys. me – medulla, cx – calyx, pa – papilla. Scale bars: 100 μm.

in the interstitial space. No staining was observed when the primary antibody was omitted.

Pax-2 expression in day 7 mouse kidney

By day 7 (d7), the number of Pax-2 positive cells in the cortex had dramatically decreased, with only a few small condensates of five or six cells and some tubular structures still displaying expression (Figure 3-3). In the medulla, Pax-2 positive cells were observed in some tubules of the inner medulla close to the calyces.

Although the number of Pax-2 positive cells in the cortex was much lower than at d1, in the papilla of d7 mice the number of Pax-2 positive cells appeared comparable to that at d1 (Figure 3-3). No staining was observed when the primary antibody was omitted even though a higher reddish background was detected compared with control in which the primary antibody had been omitted at day one.

Pax-2 expression in day 14 mouse kidney

When the kidneys of day 14 (d14) mice were analysed, the pattern of expression was found to be similar to that of d7 mice. In the cortex and medulla there were clearly fewer Pax-2 positive cells than at d1, but some Pax-2 positive cells were observed in tubular structures (Figure 3-4). In the papilla, on the other hand, the number of Pax-2 positive cells remained high and appeared comparable to that at days 1 and 7.

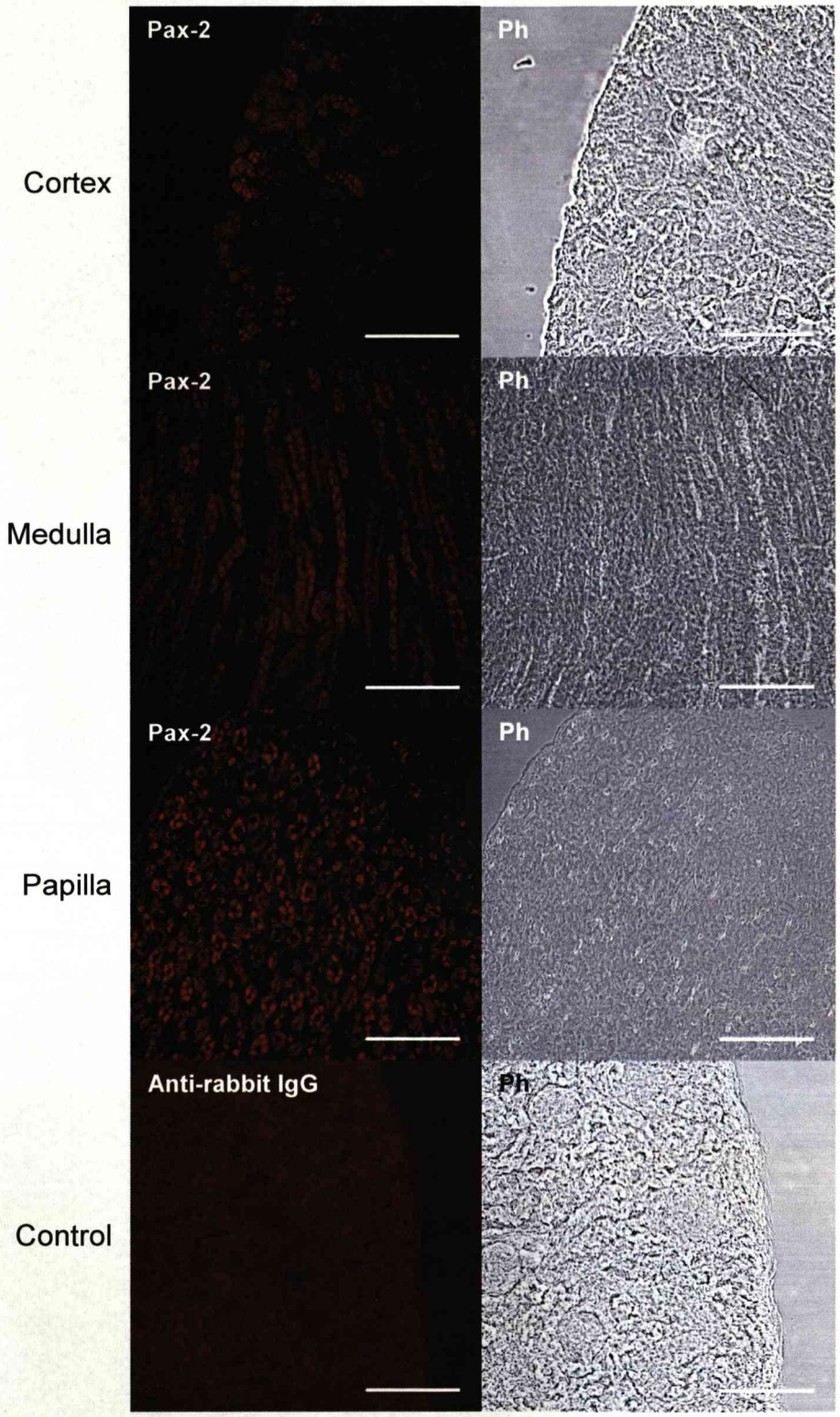


Figure 3-3 Confocal micrographs showing Pax-2 expression in frozen sections of 7 day old neonatal mouse kidney. An image of the cortex from a control staining in which the primary antibody was omitted is also shown. The images are representative of five individual kidneys. Scale bars: 100 μm.

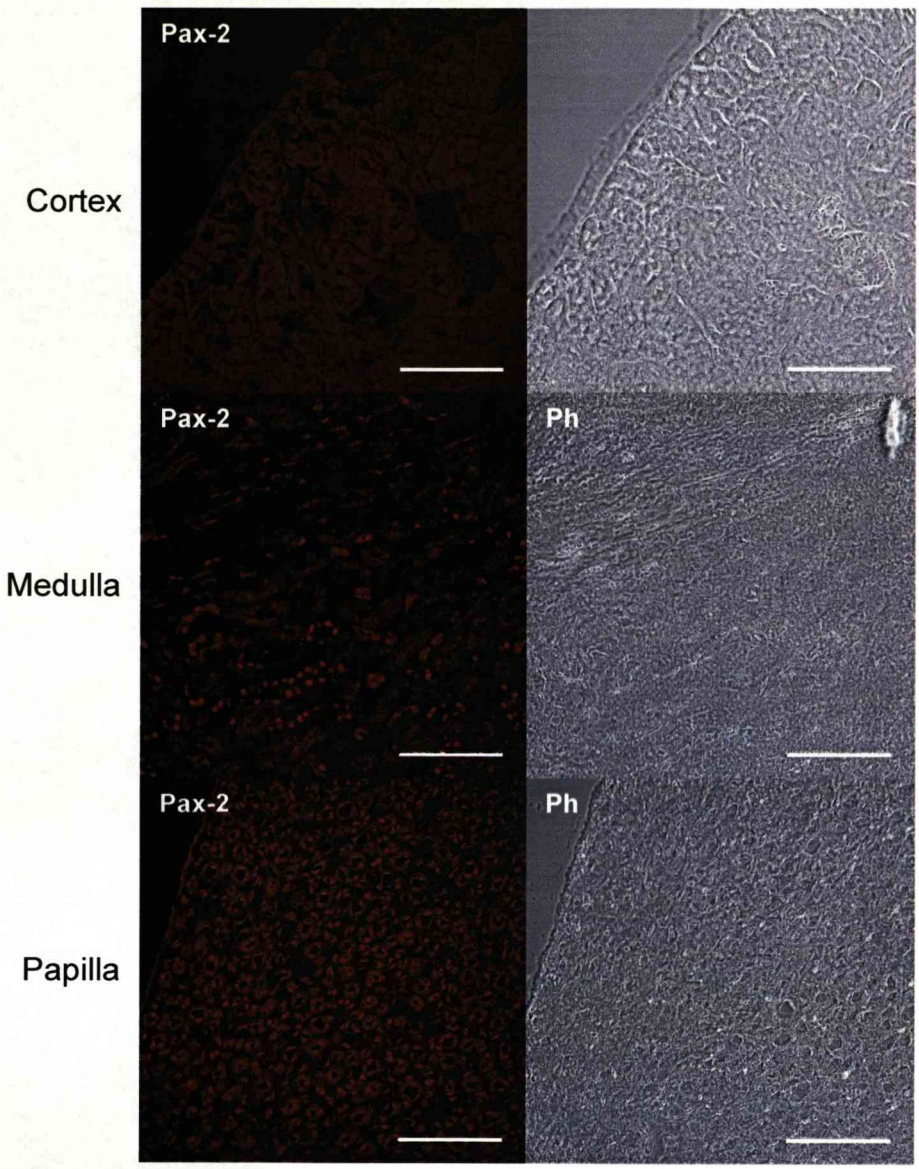


Figure 3-4 Confocal micrographs showing Pax-2 expression in frozen sections of 14 day old neonatal mouse kidney. The images are representative of five individual kidneys. Scale bar: 100 μm.

Pax-2 expression in adult mouse kidney

In adult mouse kidney very few Pax-2 positive cells were observed in the cortex and medulla (Figure 3-5). Some Pax-2 positive cells were present in the papilla, but the numbers were significantly diminished in comparison with all the previous time points. No staining was observed in a slide in which the primary

antibody had been omitted, only some non-specific red background (Data not shown).

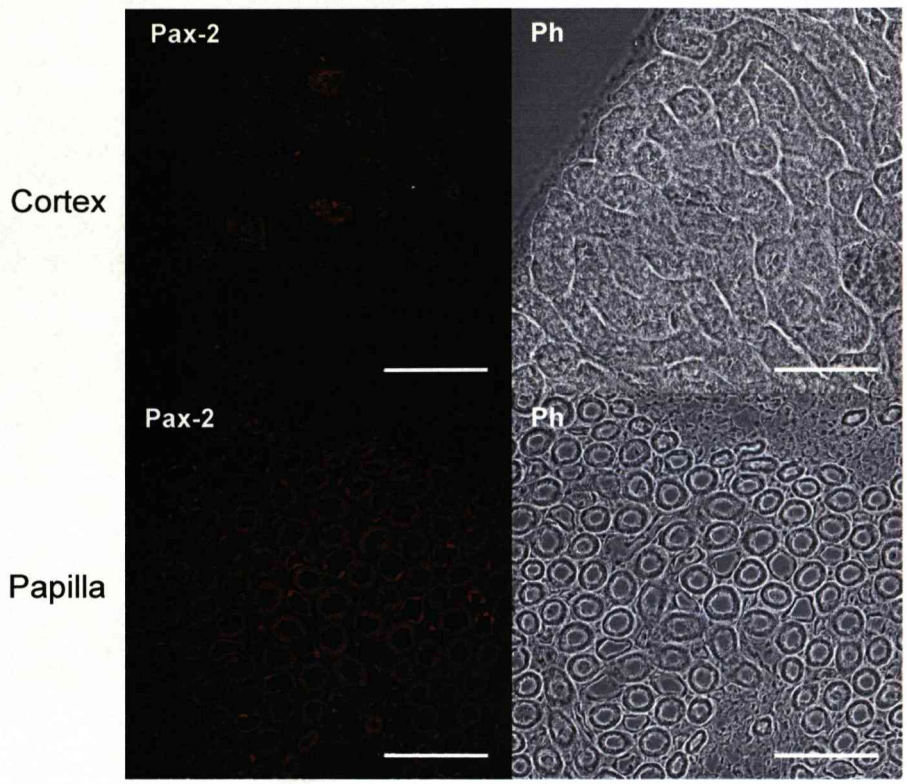


Figure 3-5 Confocal micrographs showing Pax-2 expression in frozen sections of adult mouse kidney. The images are representative of five individual kidneys. co – cortex, pa – papilla. Scale bars: 100 μm.

3.2.2 The prevalence of Pax-2 positive cells in the postnatal mouse kidney

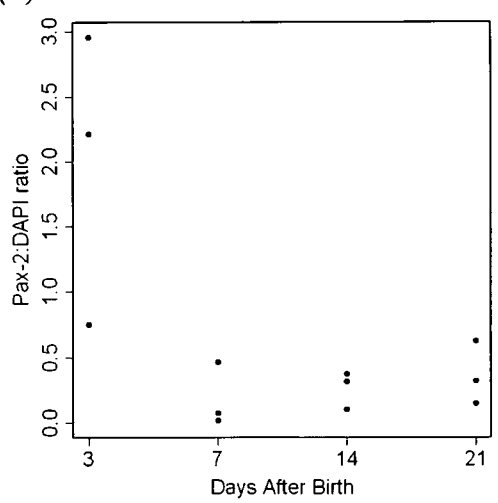
In order to study the prevalence of Pax-2 cells in postnatal mice, the ratio of all the cells (DAPI) compared with the cells expressing Pax-2 was compared between kidneys of mice at different ages. Levels of Pax-2 were studied in two different regions: the cortex-medulla and the papilla.

In the cortex-medulla (Figure 3-6), analysis of variance (ANOVA) showed a significant difference in Pax-2:DAPI ratio between the time points ($p = 0.017$). A Tukey's honest significant difference post hoc test was then applied to determine

which time points were significantly different to one another. This showed a significant difference in Pax-2 levels between days 3 and 7 (p = 0.025), when there was almost a 6 fold decrease in the mean ratio. The data also shows significant differences in cortex-medulla between day 3 and days 14 (p = 0.031) and 21 (p = 0.042). While the latter two time points showed less of a decrease in Pax-2 expression relative to day 3 than did day 7, there was no significant difference between days 7, 14, and 21 (p > 0.9).

In papilla (Figure 3-7), ANOVA showed no significant difference in Pax-2 expression levels between any time points (p > 0.05).

(a)



(b)

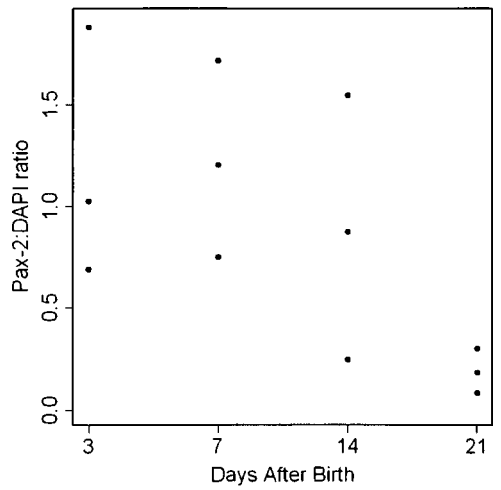
	Day 3	Day 7	Day 14
Day 7	0.025	-	-
Day 14	0.031	0.998	-
Day 21	0.042	0.981	0.996

(c)

	Mean	σ
Day 3	1.972	1.122
Day 7	0.184	0.243
Day 14	0.262	0.14
Day 21	0.364	0.239

Figure 3-6 Comparison of Pax-2:DAPI signal ratios in the cortex and medulla of kidney from mice at days 3, 7, 14, and 21. Three different slides were analysed for each kidney and three different kidneys were analysed per time point. Images were captured at 20× magnification. (a) Mean Pax-2:DAPI ratio for each kidney at each time point. (b) p values for comparison of Pax-2:DAPI ratio using Tukey's Honest Significant Difference test subsequent to significant ANOVA with p = 0.017. (c) Mean Pax-2:DAPI ratio for each time point and standard deviations (σ).

(a)



(b)

	Mean	σ
Day 3	1.2	0.616
Day 7	1.221	0.485
Day 14	0.888	0.651
Day 21	0.19	0.109

Figure 3-7 Comparison of Pax-2:DAPI signal ratios in the papilla of kidney from mice at days 3, 7, 14, and 21. Three different slides were analysed for each kidney and three different kidneys were analysed per time point. Images were captured at 20× magnification. (a) Mean Pax-2:DAPI ratio for each kidney at each time point. No significant differences between time points found with ANOVA p = 0.121. (b) Mean Pax-2:DAPI ratio for each time point and standard deviations (σ).

3.2.3 The origin of Pax-2 positive cells in the postnatal mouse kidney

WT1-Cre positive metanephric mesenchyme cells were permanently labelled with the expression of LacZ transgene and they were detected with staining for β-galactosidase. The transgenic kidneys were co-stained for β-galactosidase and Pax-2 to determine whether any of the β-galactosidase positive cells in the papilla were also expressing Pax-2. The results showed that many of the Pax-2 positive cells found in papilla in d7 mice also expressed β-galactosidase (Figure 3-8c and d). No β-galactosidase^{+}/ Pax-2^{-} cells were found in papilla.

In the cortex some β-galactosidase positive cells were found in the renal corpuscle, these cells were negative for Pax-2 (Figure 3-8a). Surprisingly, very few β-galactosidase positive cells were found in the renal tubules. Due to the metanephric mesenchymal origin of the nephron, all the renal tubules were

expected to express β-galactosidase. However, some cells contained in tubular structures in the renal cortex were Pax-2 positive even if they did not present β-galactosidase staining. Some β-galactosidase positive cells were found in tubular structures of the medulla (Figure 3-8b).

Wild type kidneys were stained for β-galactosidase to rule out any endogenous activity of the enzyme and some background was observed, however, it did not seem to be due to specific staining (Figure 3-9a). Also, staining in which the primary antibody had been omitted was performed on the transgenic kidney sections and no specific staining was observed (Figure 3-9b).

To estimate the proportion of Pax-2 positive cells expressing β-galactosidase, cells in four different fields of view using the 40× objective were counted. It was found that between 40–49% of the cells expressing Pax-2 in papilla were also expressing β-galactosidase (Table 3-1).

Table 3-1 Table summarising the proportion of Pax-2 positive cells expressing β-galactosidase in papilla. The results collected in the table were from four different fields of view on the same slide, the images were taken with a confocal microscope using the 40× immersion oil objective. In column one, the different fields of view are shown. In column 2, the number of Pax-2 positive cells counted in the field. In column three the number of β-galactosidase positive cells in the fields. In the last row, percentage of co-expression is shown.

Field of view	Pax-2 +ve	Beta gal +ve	Co-expression (%)
1	45	18	40%
2	74	36	49%
3	108	53	49%
4	126	54	42%

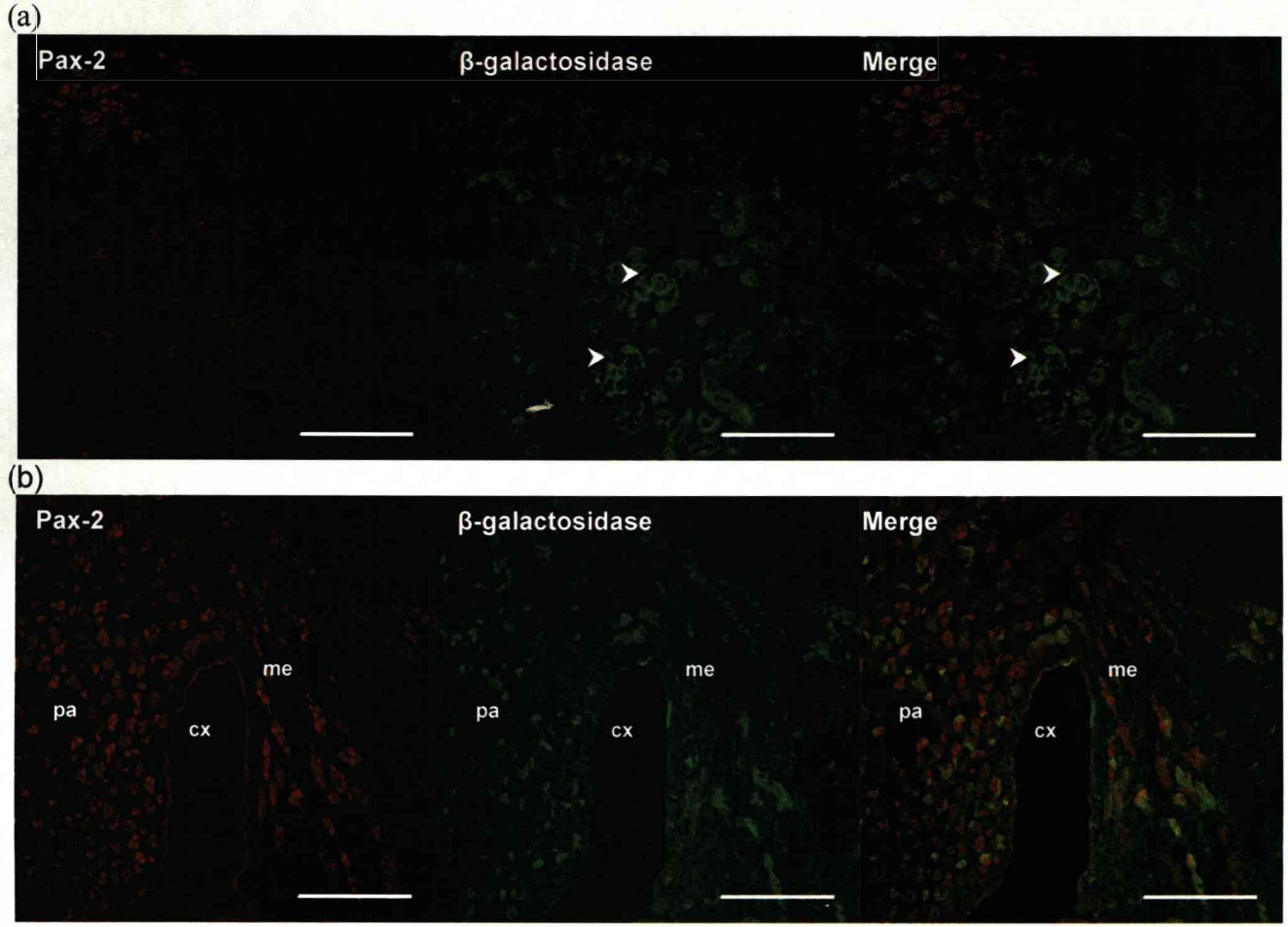


Figure 3-8(a & b) Confocal micrographs showing Pax-2 (red) and β-galactosidase (green) expression in frozen sections of 7 day old neonatal kidney from WT1 Cre transgenic mouse. (a) Renal cortex showing renal corpuscles (arrowheads). (b) Papilla and medulla. co - cortex, me - medulla, cx - calyx, pa – papilla. Scale bars: 100 μm except for (d) 20 μm. *(continues on next page)*

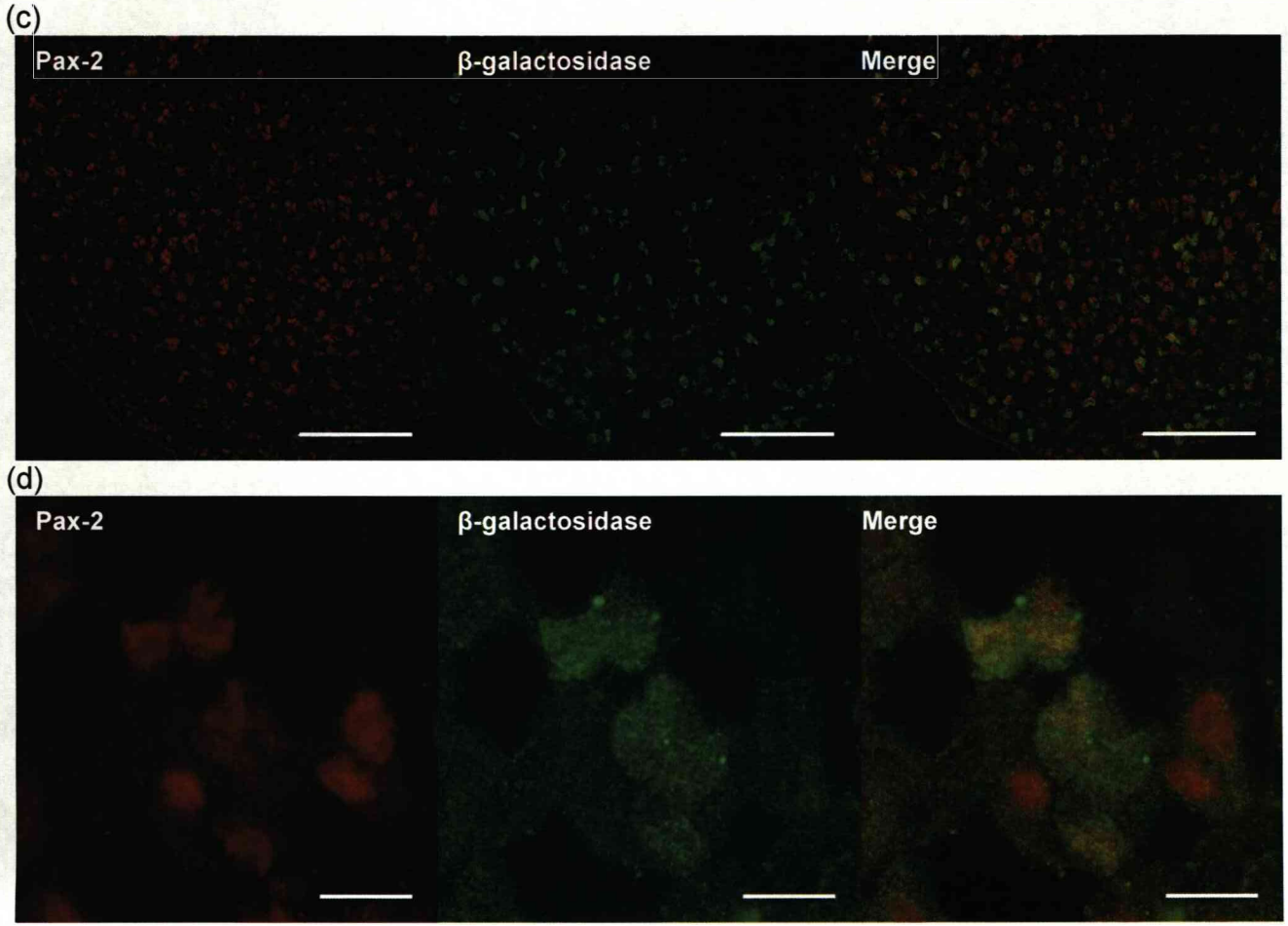


Figure 3-8 (c & d) Confocal micrographs showing Pax-2 (red) and β-galactosidase (green) expression in frozen sections of 7 day old neonatal kidney from WT1 Cre transgenic mouse. Expression levels in papilla. Scale bars: (c) 100 μm, (d) 20 μm.

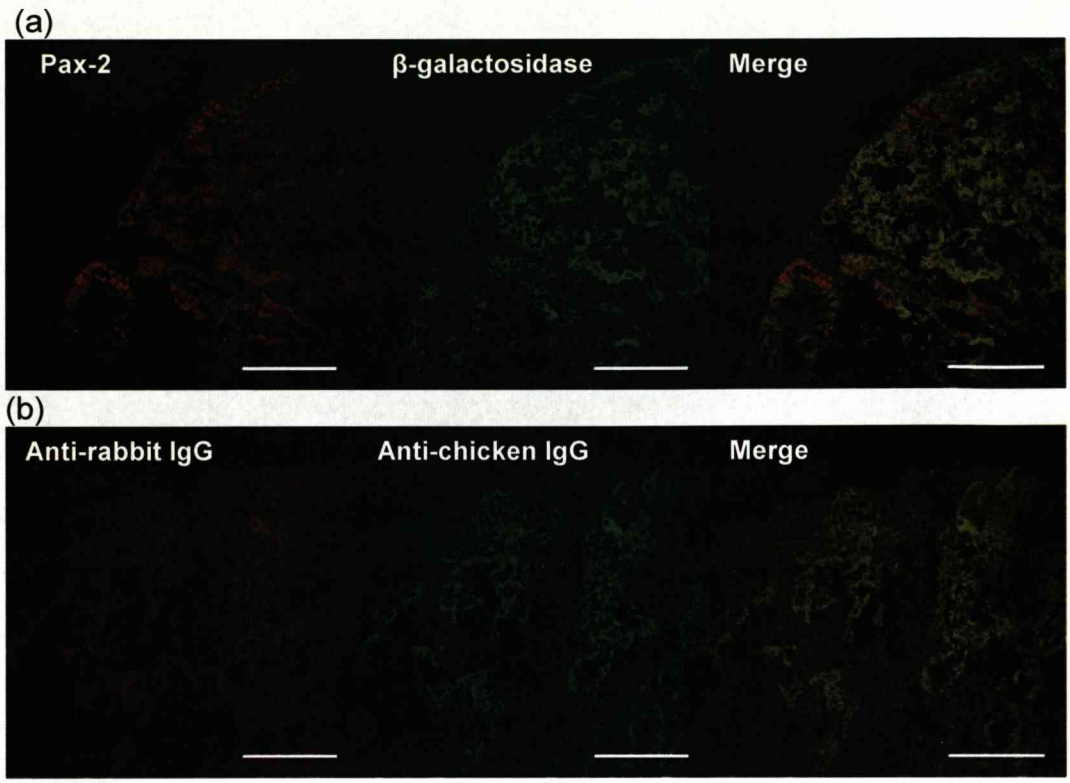


Figure 3-9 Confocal micrographs showing control slides for Pax-2 and β-galactosidase immunostainings. (a) Cortex of wild type kidney section stained for Pax-2 (red) and β-galactosidase (green). (b) Cortex of WT1-Cre transgenic mouse kidney where primary antibodies were omitted from staining; the sections were incubated with Alexa Fluor® 594 chicken anti-rabbit IgG, and AlexaFluor® 488 goat anti-chicken IgG antibodies. Scale bars 100 μm.

3.3 Discussion

In this chapter, it has been shown that a population of Pax-2 positive cells is present in the papilla of postnatal mouse kidney following the completion of nephrogenesis. It was found that the number of Pax-2 positive cells in the papilla remained constant up to three weeks following birth, but appeared to decrease in adult mice. Lineage tracing studies showed that at least half of the Pax-2 positive cells in the papilla were derived from WT1 positive cells, suggesting that their origin was the metanephric mesenchyme.

Pax-2+ cells are located in the papilla of the postnatal mouse kidney

In mature human kidney, Pax-2 was found to be expressed in a stem cell population within the renal cortex (Bussolati *et al.*, 2005). In contrast, this study showed that in mature mouse kidney, Pax-2 positive cells were almost exclusively present in the renal papilla. Although this seems to suggest that the distribution of Pax-2 positive cells in mature human and mouse kidney is different, however, the papilla of human kidney has not yet been analysed for the presence of Pax-2 positive cells. Furthermore, the human kidney samples analysed in the Bussolati study comprised regions of apparently 'normal' tissue excised from kidneys afflicted with renal carcinoma. It is therefore possible that Pax-2 positive stem cells present in the cortex of these human kidneys might have arisen from the renal carcinoma, for it has been shown that Pax-2 is expressed in the majority of clear cell renal carcinomas, which is the most common form of renal cancer (Mazal *et al.*, 2005). It is difficult to perform a detailed study of the distribution of Pax-2 positive cells in normal human kidney because of the high demand of such organs for transplantation. In future, it would be interesting to investigate the distribution of Pax-2 positive cells in non-human primate and porcine kidney to determine if the expression profile of this protein in mouse is similar to that of other mammals.

A hypothesis of the current study is that the Pax-2 positive cells present in the papilla of the postnatal mouse kidney represent a stem cell population. Oliver *et al.* (2004) found a population of low rate cycling cells in the renal papilla, which they suggested were possible stem cells. In contrast with the present findings the

majority of these cells were located in the interstitium, with some in tubular epithelial cells. On the other hand, Maeshima *et al.* (2006; 2003) identified a similar population of low rate cycling cells present in different fractions of renal tubules, rather than in the interstitium. The difference in location between these papers could be explained by the differences in the protocol used for BrdU labelling, by the age of the animals, or even by the species.

Among the Pax-2 positive cells in the papilla, different levels of expression were observed. A previous report had shown that Pax-2 is re-expressed in proximal tubules after damage, and these Pax-2 expressing cells have the potential to dedifferentiate and proliferate in order to replace the damaged tubular cells (Imgrund *et al.*, 1999). Therefore, one possible explanation for the differences in Pax-2 levels in the papilla is that expression levels could be related to the differentiation state of the cells. Cells with higher levels of Pax-2 expression may be in a less differentiated state than those presenting lower expression, and could therefore have more differentiation potential.

The number of Pax-2 positive cells in the mouse papilla is decreased in the adult

In this study, the number of Pax-2 positive cells in the papilla appeared to be reduced compared with 3 week old adolescent mice. If the Pax-2 positive cells are indeed stem cells, the reduced numbers in the adult might suggest that the number of stem cells reduces with age. The adults used in this study were young adults of about 8-10 weeks. Although beyond the scope to this study, it would be interesting in future to determine the number of Pax-2 positive cells at 6 months

and 12 months to investigate the effect of ageing on the number of Pax-2 positive cells. However, it is interesting to note that Cai *et al.* (2005) showed that a high number of Pax-2 positive cells were present in the papilla of adult mouse kidney. Therefore, it is possible that the reduced number of Pax-2 positive cells observed here could have been due to technical problems related to the fixation procedure, given the fact that the size of the kidneys was significantly bigger than at the earlier time points and so penetrability of the fixating agent could have had an adverse effect on subsequent staining.

Lineage tracing of Pax-2 positive cells

The renal papilla is the region of the kidney where the collecting tubules empty urine into the renal pelvis. Therefore, although this region contains the tips of the some of the loop of Henle, most of the tubules are collecting tubules derived from the UB. In early kidney development, Pax-2 is expressed in both MM and in the UB. Although MM cells can give rise to UB cells and their derivatives, it has been established that UB cells have a more limited potential and cannot give rise to the cells of the nephron (Qiao *et al.*, 1995). It was therefore important to confirm that at least some of the Pax-2 positive cells in the papilla were derived from MM rather than UB. The results showed that 40-50% of Pax-2 positive cells were derived from WT1-expressing cells. Given that in the developing kidney, the MM but not the UB expresses WT1, this result shows that the Pax-2^+/β-galactosidase$^+$ cells cannot be derived from UB. The most likely interpretation is that the Pax-2^+/β-galactosidase$^+$ cells are derived from MM, although it is also

possible that they could be derived from a non-renal population of WT1-expressing cells that populates the kidney during its development.

Approximately half of the Pax-2 positive cells found in papilla did not express β-galactosidase. This may suggest that these cells had a UB origin, or it may have been that they were expressing β-galactosidase below detection levels. However, in the cortex, although β-galactosidase positive cells were found in the renal corpuscle, few positive cells were observed in the renal tubules of the nephron. This result was unexpected because the whole nephron is derived from WT1 positive MM cells. The absence of β-galactosidase staining in all parts of the nephron is most likely due to incomplete Cre penetrability.

In this chapter, a population of Pax-2 positive cells was identified in the papilla of postnatal mouse kidney; a region that has been postulated to be a niche for rodent renal stem cells Oliver *et al.* (2004). A hypothesis of this thesis is that these Pax-2 positive cells are a population of kidney stem cells. To test this hypothesis, it is necessary to show that the Pax-2 positive cells have the ability to self-renew and differentiate to renal-specific cell types. In order for these properties to be assessed, the cells must be isolated from the kidney and expanded *in vitro*. In the following chapter, a method is developed to enable the Pax-2 positive cells to be identified using a cell surface marker and isolated using Magnetic Activated Cell Sorting.

Chapter 4: Isolation of putative KSC using MACS

4.1 Introduction

In the previous chapter a population of Pax-2 expressing cells was identified in the papilla of postnatal mouse kidney. To further study these Pax-2 positive cells and evaluate whether or not they exhibited properties consistent with stem cells, it was necessary to first separate them from the rest of the kidney. A variety of different techniques can be used for cell sorting and separation of a particular population of cells from a tissue, however, two of the most commonly used techniques are fluorescence-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS) (Pappas and Wang, 2007).

FACS has been used for many years to identify and separate rare cells. However, as FACS individually sorts each cell within the population the number of cells that can be processed is limited. This means that it is inefficient at isolating very rare cells from a large population of cells (Radbruch and Recktenwald, 1995). MACS can be used to process a high number of cells obtaining significant numbers of the population of interest (Radbruch and Recktenwald, 1995). MACS is therefore the more appropriate technique for the isolation of rare cells within a population, and thus is the preferred technique for the isolation of stem cells, which typically represent a small fraction of the cells within a tissue (Buageaw *et al.*, 2005; Pappas and Wang, 2007). MACS has been successfully used to isolate amniotic fluid stem cells (De Coppi *et al.*, 2007), liver progenitor cells (Qin *et al.*,

2004), epithelial stem cells (Collins *et al.*, 2001), and human kidney progenitor cells (Bussolati *et al.*, 2005; Sagrinati *et al.*, 2006).

As with FACS, the MACS strategy separates cells using specific markers, however in contrast to FACS, which sorts cells one-by-one, MACS separation can be performed on large number of cells simultaneously. Briefly, the whole population of cells from which a specific fraction is to be isolated is typically incubated with magnetic beads that have an antibody or a lectin bound to their surfaces, which will tag the cell population of interest. The cells are then passed through a column placed in a magnetic field and the cells tagged with magnetic beads are retained in the column while unlabelled cells pass through (Lanza *et al.*, 2006). While this technique allows for the more rapid processing than FACS, MACS specifically requires cell surface markers unlike FACS, which can use any cellular marker– surface markers or intracellular markers, such as transcription factors – that can be fluorescently tagged.

The selection of a particular fraction of cells by magnetic cell sorting can be achieved in two ways. The first one is positive selection, in which the population of interest is labelled with magnetic beads. When the unlabelled cells have passed through the magnetic field, the column is removed from the magnetic field and the cells of interest are collected.

The alternative approach is a negative selection, which uses an antibody that tags the unwanted cells causing them to be retained in the column and allowing only the desired cell fraction to pass through. The advantage of negative selection is that the cells of interest are not bound to magnetic beads. A combination of

positive and negative selection is very effective for enriching stem cells from a mixed population, and facilitates the isolation of a pure population (Lanza *et al.*, 2006).

Using the stem cell marker CD133 two different groups have been able to isolate a multipotent population of cells from human kidney with MACS positive selection (Bussolati *et al.*, 2005; Sagrinati *et al.*, 2006). Other groups have performed MACS negative selection in order to enrich a population of mesenchymal stem cells from bone marrow by depleting unwanted hematopoietic cells (Morigi *et al.*, 2004; Rombouts and Ploemacher, 2003).

The cell population of interest in this study expressed the nuclear transcription factor Pax-2. However, Pax-2 is unsuitable for MACS given its nuclear location and the stem cell marker CD133, as used by Camussi and Romagnani groups (Bussolati *et al.*, 2005; Sagrinati *et al.*, 2006), is not suitable in mice as they lack the antigen recognized by the AC133 monoclonal antibody. Therefore, in order to separate these cells from the total population of the kidney using MACS, it would be necessary to identify an alternative cell surface antigen specific to this cell population.

Complex carbohydrates are present on the surfaces of all cells, where they are essential components of glycoproteins, glycolipids and proteoglycans (Sharon and Lis, 1989). Cell surface carbohydrates play important roles in cell-cell recognition and cell adhesion (Brandley and Schnaar, 1986). Lectins are a group of glycoproteins that are involved in a wide range of biological processes

including cell adhesion and immune function. Lectins specifically bind to particular cell surface carbohydrates (Scarpino *et al.*, 1998).

Based on their properties for binding carbohydrate residues, lectins can be used for cell isolation (Scarpino *et al.*, 1998). Lectins have been used to isolate different cell types such as liver endothelial cells using MACS (Rodes and Milton, 1998) or Sertoli cells using lectin coated dishes (Scarpino *et al.*, 1998). Lectins have also been used for enriching hematopoietic progenitors from bone marrow using lectin coated devices (Rodes and Milton, 1998).

Several groups have studied lectin binding patterns in developing and adult mouse kidney using immunostaining (Hanai *et al.*, 1994; Holthofer, 1983; Laitinen *et al.*, 1987; Schulte and Spicer, 1983). Changes in glycoconjugate composition have been observed during mouse kidney development using lectins (Hanai *et al.*, 1994). However, there is a specific regional distribution of glycoconjugates in adult mammalian kidney, which shows the functional compartmentalization of the organ (Laitinen *et al.*, 1987).

Hanai *et al.* (1994) studied the binding pattern of some lectins during mouse kidney development. This group showed that peanut agglutinin lectin (PNA) did not bind any structures in prenatal mouse kidney and in postnatal mouse, and that from newborn to senescent mouse, PNA binding was restricted to proximal tubules. They also showed that *Dolichos biflorus* agglutinin (DBA) lectin is expressed in collecting duct in prenatal mouse and newborn, but is absent in adult and senescent mouse. When they studied *Phaseolus vulgaris* leucoagglutinin

(PHA-L), there was no lectin binding in prenatal mouse and the lectin bound the proximal tubules and glomeruli from newborn onwards.

Laitinen *et al.* (1987) performed a lectin binding study in embryonic and in adult mouse kidney. PNA binding in adult kidney showed similar results to the study performed by Hanai *et al.* (1994). PNA bound mainly to proximal tubules, and some staining was also observed in collecting ducts. In embryonic kidney, PNA was found to bind most of the structures in developing kidney, including the ureteric bud, primary cell condensates, and the S-shaped body. However uninduced mesenchyme and maturing glomeruli later in development did not show PNA lectin binding. In this study, DBA lectin was also found to bind collecting duct during early development and in adult kidneys (Hanai *et al.*, 1994).

Laitinen *et al.* (1987) also studied succinylated wheat germ agglutinin (sWGA) and *Griffonia simplicifolia* lectins (GSL-I). sWGA lectin was found to bind all structures in developing kidney, but in adult was largely restricted to proximal tubules with some binding also detected in distal tubules and collecting ducts. GSL-I was found to bind endothelial structures in the kidney.

As a number of lectins have already been shown to bind to specific cell populations within the rodent kidney, it was decided that a lectin, rather than antibody-based screening approach, would be more likely to yield a suitable cell surface marker for the Pax-2 positive cells in the papilla.

4.1.1 Aims

In this chapter, the isolation of Pax-2 positive cells from neonatal mouse kidney using MACS is reported. In order to facilitate this isolation it was necessary to identify a suitable a cell surface marker co-expressed with the nuclear marker Pax-2.

The first aim was to co-stain postnatal mouse kidney with Pax-2 and a panel of 21 lectins. Following identification of a lectin that bound specifically to the Pax-2 positive cells, the next aim was to investigate whether magnetic beads conjugated to the lectin could be used to isolate the Pax-2 positive cell population.

4.2 Results

4.2.1 Lectin binding study in neonatal kidneys during development

Some of the 21 lectins screened at different stages of kidney development did not show specific staining patterns in the kidney and were discarded (Table 4-1).

Nine lectins were selected for further study because they appeared to bind specific structures in the kidney. However, only six appeared as good candidates for Pax-2 positive cell isolation and were therefore further analysed. The lectins selected for deeper analysis were PNA, DBA, sWGA, PHA-L, GSL-I and *Solanum tuberosum* lectin (STL).

Some of these lectins bound the surface of Pax-2 positive cells in the developing kidney while others bound the surface of cells negative for Pax-2. PNA, DBA and STL lectins seemed to bind the surface of some Pax-2 positive aggregates and tubular structures in renal cortex, whereas sWGA and PHA-L bound the surface

Table 4-1(a) Lectins used in screening to find a candidate to isolate Pax-2 cells using MACS. The table shows lectin name, common name, the sugar specificity, and the expression pattern observed when kidneys from one day old mice were stained with the lectins (Sumar N. *et al.*, 1993; Vector laboratories, 2009). *(continued on next page)*

Lectin	Common name	Sugar specificity	Binding pattern
Arachis hypogaea	Peanut (PNA)	Galactose, N-acetylgalactosamine	Tubular structures in cortex, medulla and papilla.
Artocarpus integrifolia	Jacalin	galactosyl (β 1,3) N-acetylgalactosamine	Non specific staining
Canavalia ensiformis	Concanavalin A (ConA)	α-mannose, α-glucose	Non specific staining
Datura stramonium	Thorn apple (DSL)	(β 1,4) N-acetylglucosamine	Non specific staining
Dolichos biflorus	Horsegram (DBA)	α-N-acetylgalactosamine	Tubular structures in cortex, medulla and papilla.
Erythrina cristagalli	cockspur coraltree (ECL)	(β 1,4) N-acetylglucosamine	Non specific staining
Glycine max	Soybean agglutinin (SBA)	N-acetylgalactosamine	Tubular structures in cortex, medulla and papilla.
Griffonia simplicifolia	(GSL I)	α-N-acetylgalactosamine	glomeruli, endothelium, papillary structures
Griffonia simplicifolia	(GSL II)	N-acetylglucosamine	Non specific staining
Lens culinaris	lentil lectin (LCA)	α-mannose, α-glucose	Non specific staining
Lycopersicon esculentum	Tomato (LEL)	N-acetylglucosamine	Tubular structures in cortex, medulla and papilla.

Table 4-1(b) Lectins used in screening to find a candidate to isolate Pax-2 cells using MACS. The table shows lectin name, common name, the sugar specificity, and the expression pattern observed when kidneys from one day old mice were stained with the lectins (Sumar N. *et al.*, 1993; Vector laboratories, 2009).

Lectin	Common name	Sugar specificity	Binding pattern
Phaseolus vulgaris	red kidney bean erythroagglutinin (PHA-E)	N-acetylgalactosamine	Non specific staining
	red kidney bean leucoagglutinin (PHA-L)	N-acetylgalactosamine	Tubular and glomerular structures in cortex and medulla. No binding present in papilla
Pisum sativum	Garden pea (PSA)	α-mannose	Non specific staining
Ricinus communis	Castor bean (RCA)	N-acetylgalactosamine	Non specific staining
Solanum tuberosum	Potato (STL)	N-acetylglucosamine	Tubular structures in cortex, medulla and papilla
Sophora japonica	Pagoda tree (SJA)	N-acetylgalactosamine, galactose	Non specific staining
Triticum vulgaris	Wheat germ agglutinin (WGA)	N-acetylglucosamine, sialic acid	Non specific staining
Triticum vulgaris	succynilated wheat germ (sWGA)	N-acetylglucosamine	Tubular structures in cortex, medulla and papilla.
Ulex europaeus	Gorse (UEA I)	α-fucose	Non specific staining
Vicia villosa	Hairy vetch (VVL)	N-acetylgalactosamine	Most of the tubular structures

of most of the cells in cortex negative for Pax-2. GSL-I presented a different pattern, binding glomeruli and endothelial structures. All these lectins bound the surface of most of the Pax-2 positive cells in the papilla with the exception of PHA-L where no lectin binding was observed. PNA was the only lectin that specifically bound most of the Pax-2 positive cells at all life stages tested, therefore, this lectin was bound to magnetic beads in order to isolate renal cells using MACS.

Peanut agglutinin lectin (PNA)

In the renal cortex and medulla of one day old mice PNA lectin clearly bound tubular structures, some of which contained Pax-2 positive cells (Figure 4-1a). However, the PNA lectin also bound the surface of tubular structures that did not contain Pax-2 positive cells. PNA also bound the surface of some of Pax-2 positive cells in the comma- and S-shaped bodies, and Pax-2 positive cells that were condensed around the tips of the lectin binding tubules, but in these cases staining was considerably weaker than in tubular structures.

PNA lectin bound the surface of most of the cells expressing Pax-2 present in the renal papilla (Figure 4-1a). The lectin seemed to bind the surface of the tubules expressing Pax-2 and the surface of one or two cells or small clusters of cells.

In 7 day (Figure 4-2a), 14 day, and adult mice the amount of PNA binding apparent in the cortex decreased significantly with only a few tubules still binding PNA (Data not shown). In contrast the numbers of cells still binding PNA in the papilla remained relatively high, with the lectin still binding the majority of Pax-2

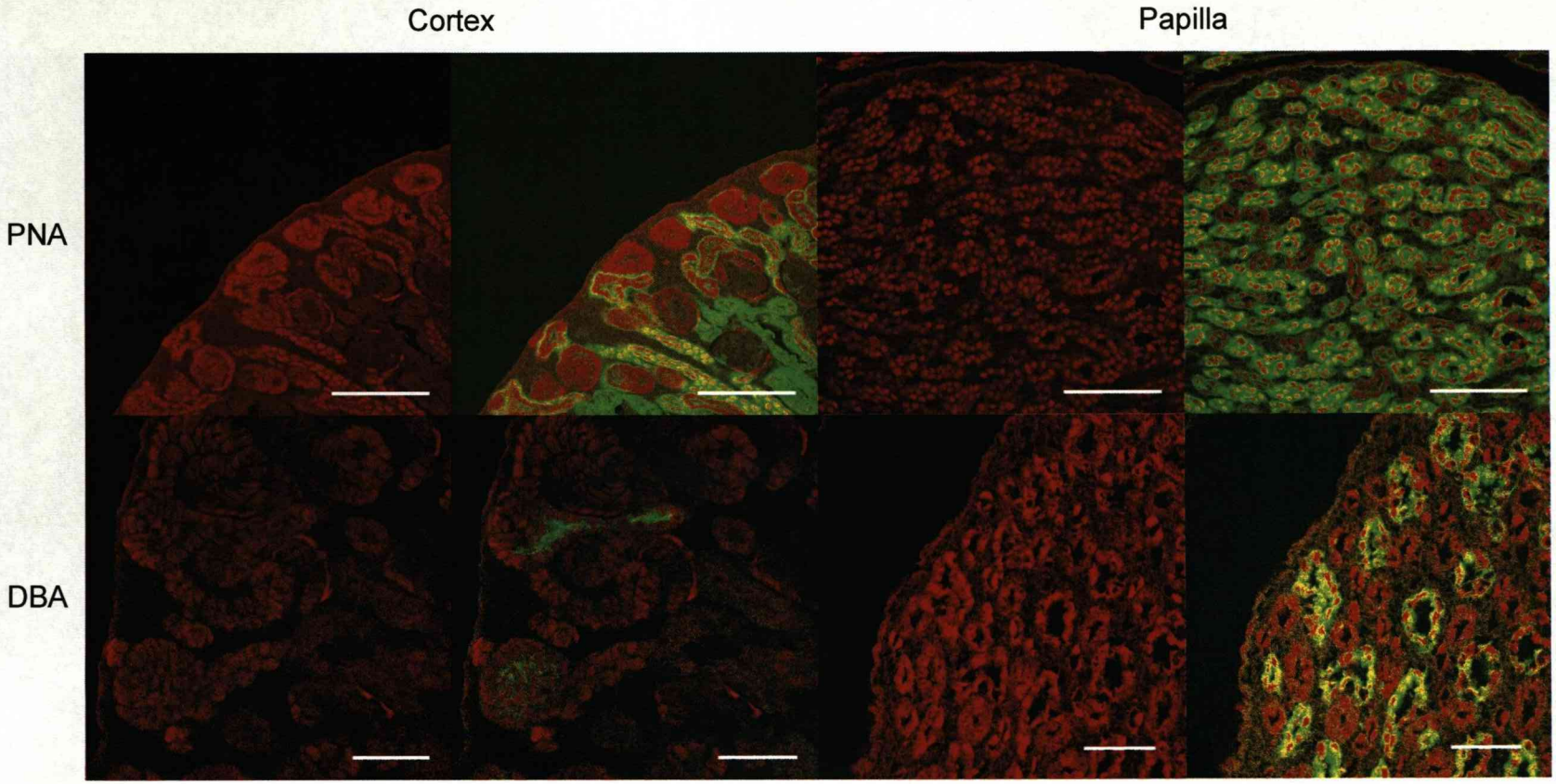


Figure 4-1(a) Micrographs showing the expression of Pax-2 (red) and the binding of the lectins PNA and DBA (green) in the cortex and papilla of kidneys from 1 day old mice. Scale bars: 100 μm. *(continued on next page)*

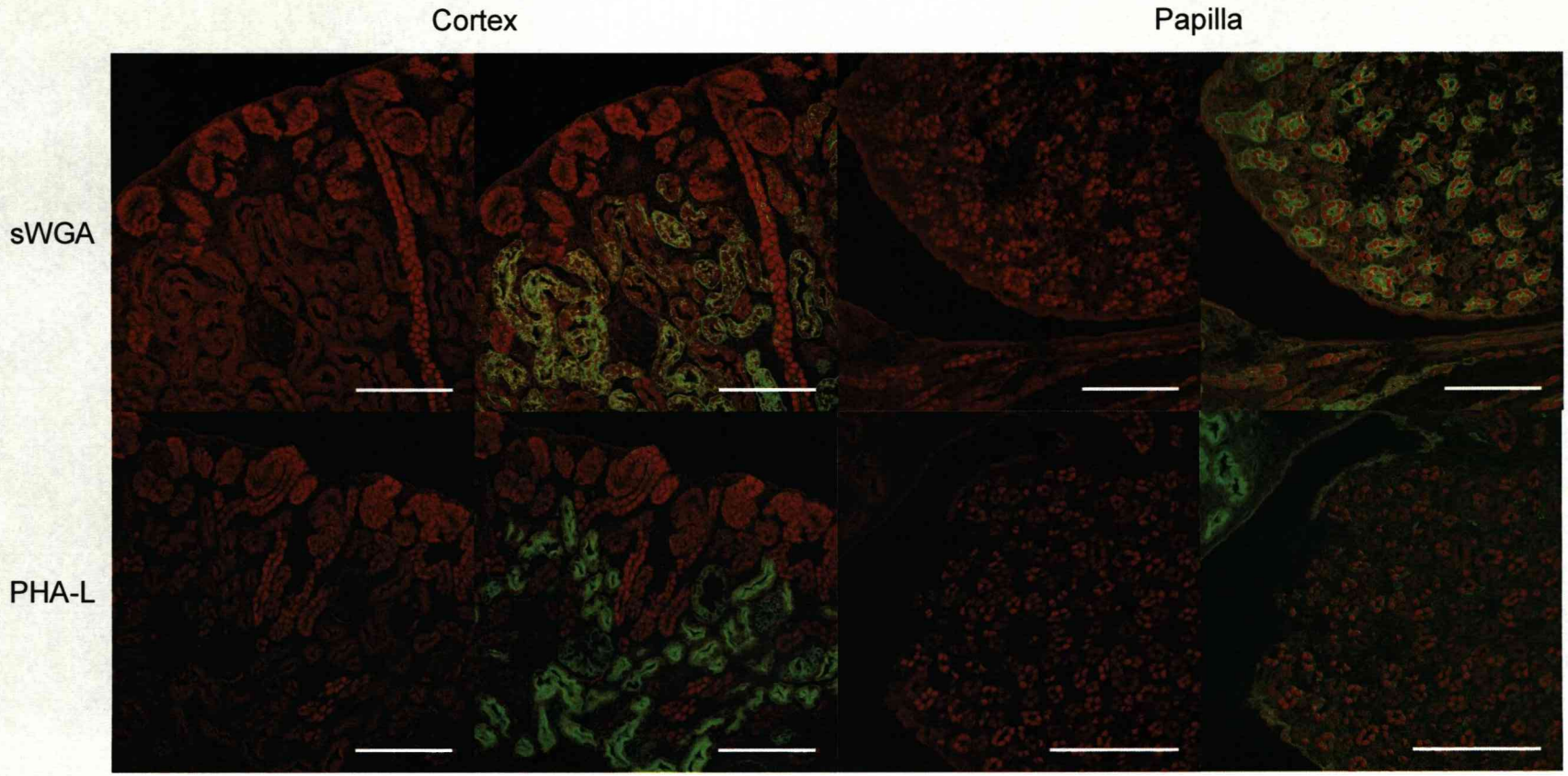


Figure 4-1(b) Micrographs showing the expression of Pax-2 (red) and the binding of the lectins sWGA and PHA-L (green) in the cortex and papilla of kidneys from 1 day old mice. Scale bars: 100 μm. ***(continued on next page)***

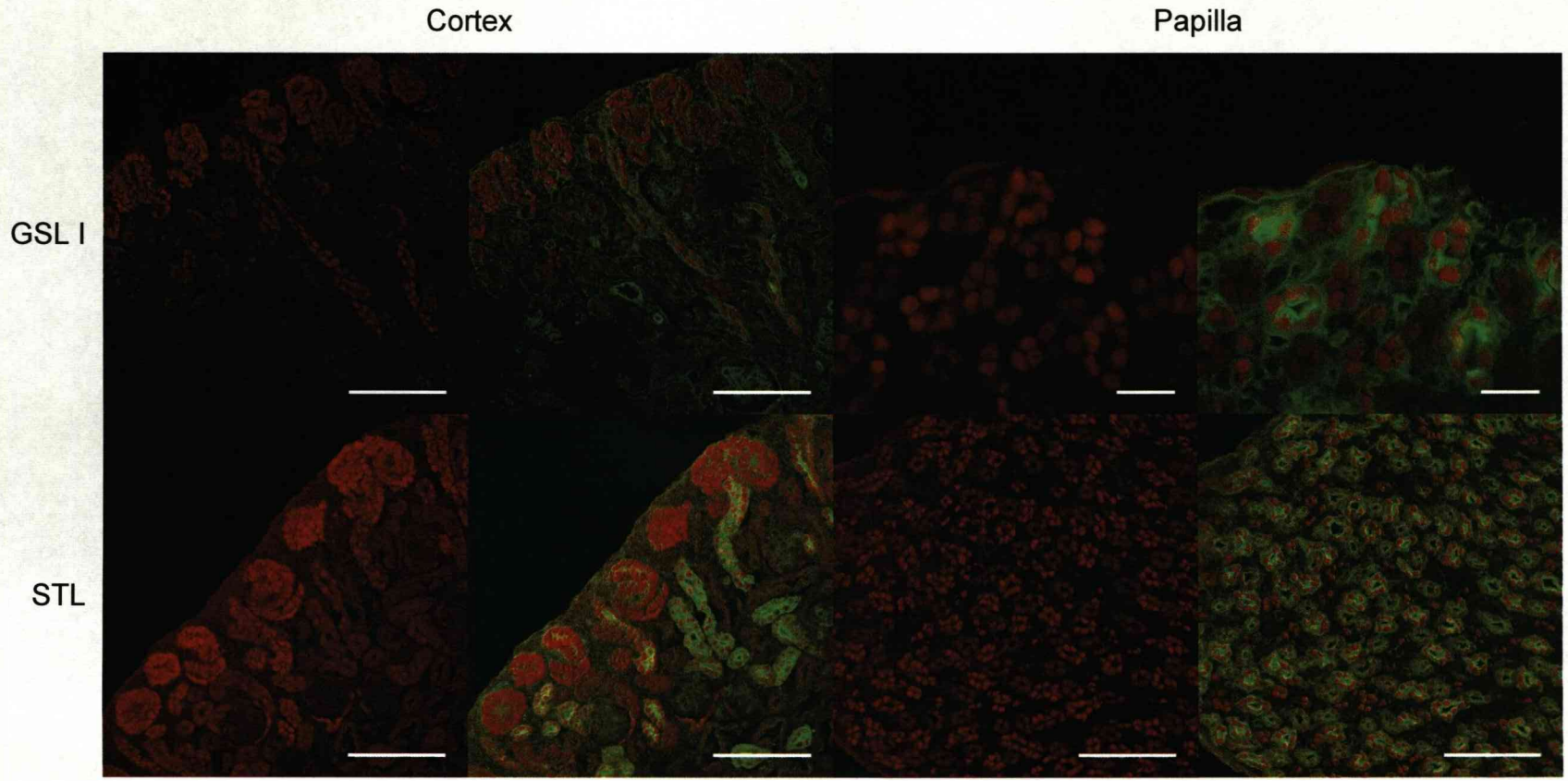


Figure 4-1(c) Micrographs showing the expression of Pax-2 (red) and the binding of the lectins GSL-I and STL (green) in the cortex and papilla of kidneys from 1 day old mice. Scale bars: 100 μm, except GSL-I papilla – 20 μm.

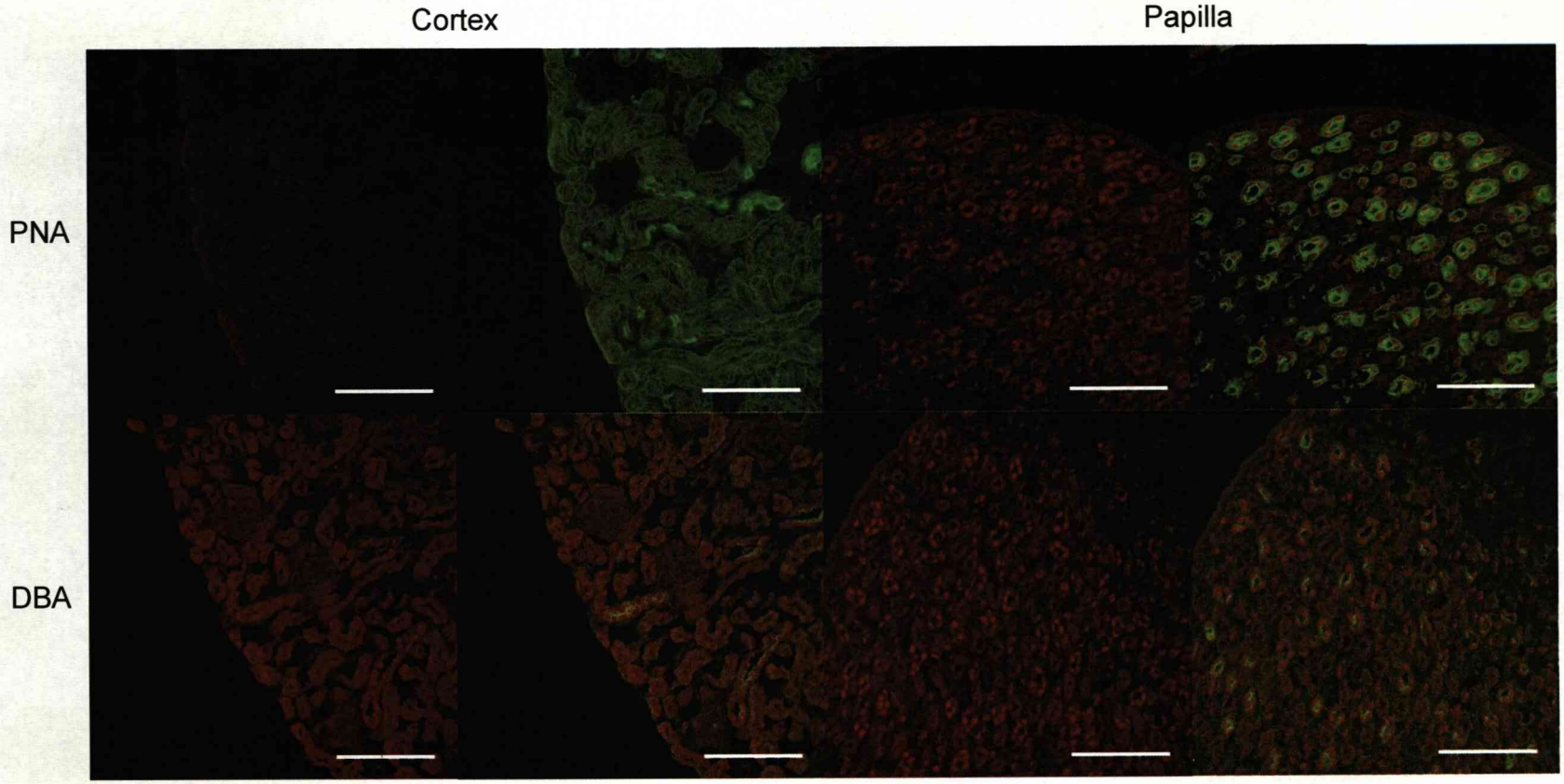


Figure 4-2(a) Micrographs showing the expression of Pax-2 (red) and the binding of the lectins PNA and DBA (green) in the cortex and papilla of kidneys from 7 day old mice. Scale bars: 100 μm. *(continued on next page)*

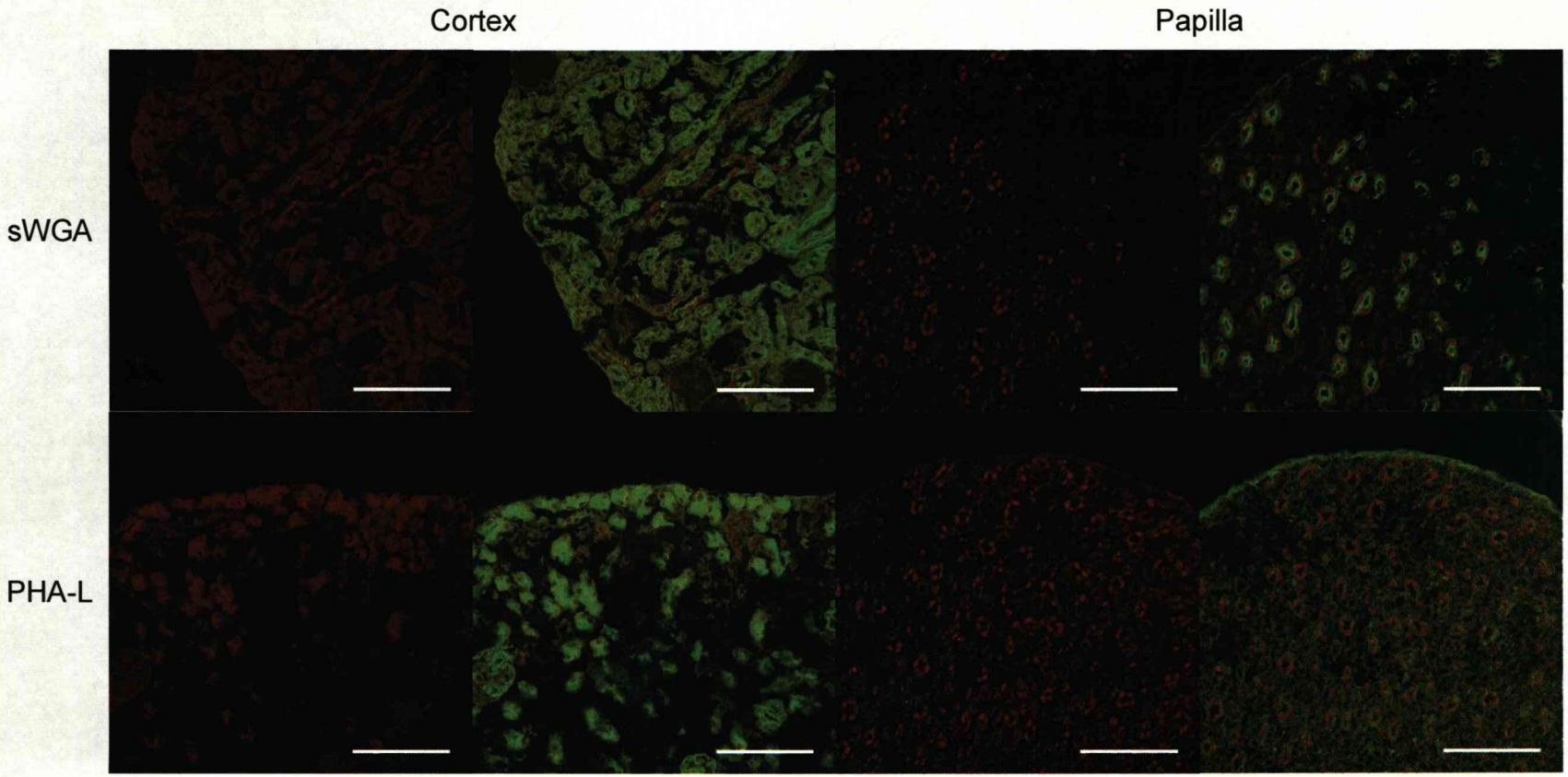


Figure 4-2(b) Micrographs showing the expression of Pax-2 (red) and the binding of the lectins sWGA and PHA-L (green) in the cortex and papilla of kidneys from 7 day old mice. Scale bars: 100 μm. *(continued on next page)*

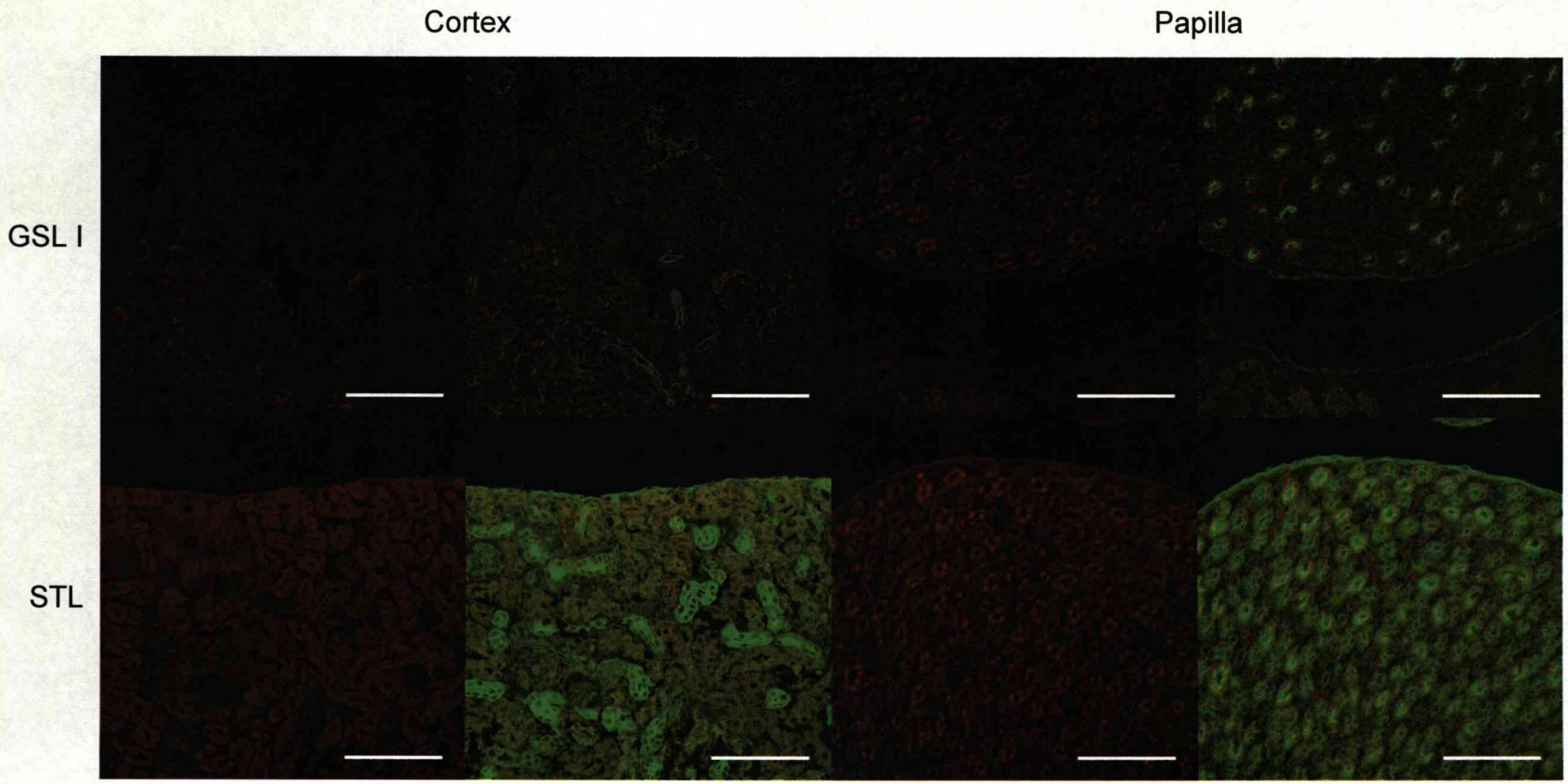


Figure 4-2(c) Micrographs showing the expression of Pax-2 (red) and the binding of the lectins GSL-I and STL (green) in the cortex and papilla of kidneys from 7 day old mice. Scale bars: 100 μm.

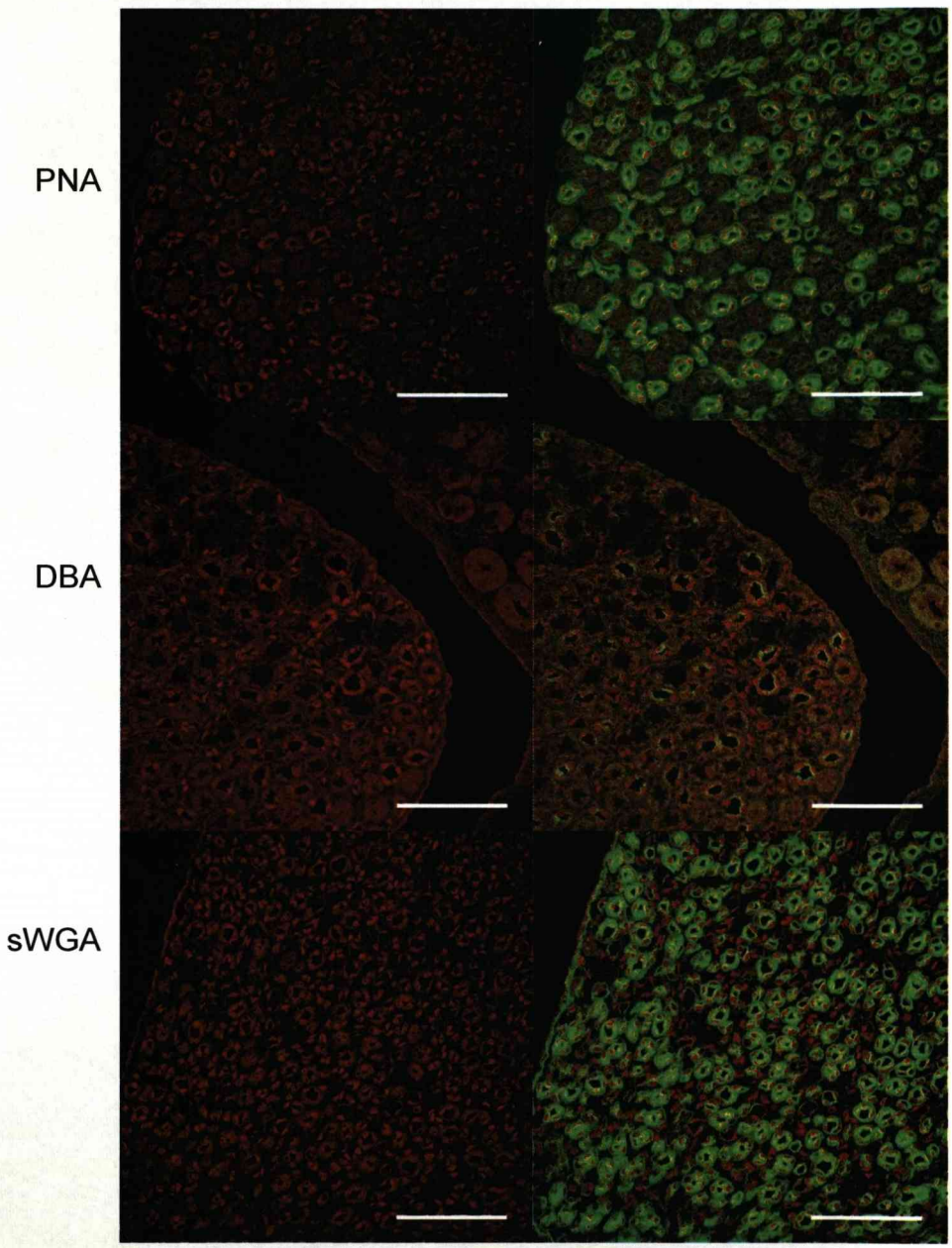


Figure 4-3 Micrographs showing the expression of Pax-2 (red) and the binding of PNA, DBA, and sWGA (green) in the cortex and papilla of kidneys from 14 day old mice. Scale bars: 100 μm

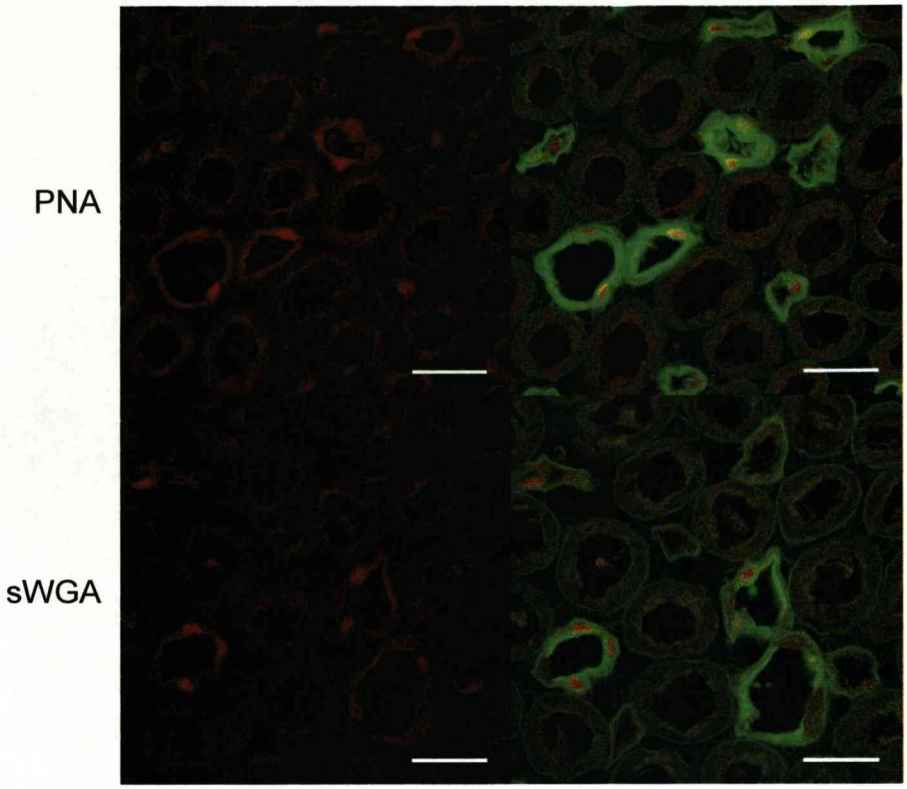


Figure 4-4 Micrographs showing the expression of Pax-2 (red) and the binding of PNA, and sWGA (green) in the cortex and papilla of kidneys from adult mice. Scale bars: 20 μm.

positive cells, predominantly those in tubules (Figure 4-2a, Figure 4-3 and Figure 4-4).

Dolichos biflorus agglutinin (DBA)

DBA lectin was found to bind the surface of some Pax-2 positive cells in the cortex and medulla at day 1 (Figure 4-1a). However, only a few tubules expressing Pax-2 in the cortex and medulla were found to have lectin staining. In 7 day old mice some DBA binding was still seen in the cortex and medulla (Figure 4-2a), however, by day 14 and in adult mice there was no DBA binding (Data not shown).

In the renal papilla of one day old mice, the lectin appeared to bind the surface of many tubules containing Pax-2 positive cells (Figure 4-1a). By visual analysis there seemed to be fewer tubules in the papilla positive for DBA binding than there were for PNA. Very little DBA binding was evident in the papilla of day 7 (Figure 4-2a), day 14 (Figure 4-3), and adult mice (Data not shown).

Succinylated wheat germ agglutinin (sWGA)

When kidneys of one day old mouse were stained with sWGA strong staining was observed in some tubular structures of the cortex (Figure 4-1b). These tubules did not contain Pax-2 positive cells. No lectin staining was observed in renal corpuscles, comma- and S-shaped bodies, or any Pax-2 expressing cells in the nephrogenic area. In the medulla the sWGA lectin bound many tubules, and some of these tubules contained Pax-2 positive cells. At days 7 (Figure 4-2b) and 14 and in adult sWGA bound the majority of tubules in the cortex but these showed very little Pax-2 expression (Data not shown).

When renal papilla was analysed it was found that the lectin bound the surface of the majority of tubules present in papilla containing cells expressing Pax-2 at all time points (Figure 4-2b, Figure 4-3 and Figure 4-4).

Phaseolus vulgaris leucoagglutinin (PHA-L)

The lectin PHA-L in one day old neonates appeared to bind the sugars on the surface of some tubules and glomerular and developing glomerular structures (Figure 4-1b). However, the lectin binding seemed to be absent in the nephrogenic zone, where Pax-2 was expressed. Some tubules in the inner medulla

presented lectin staining, but they did not contain Pax-2 positive cells. At later time points the lectin bound the majority of structures in the cortex and medulla, but, again, none of these structures contained significant numbers of Pax-2 positive cells (Figure 4-2b).

The lectin did not bind the surface of any of the Pax-2 positive cells in the papilla at any time point (Figure 4-1b and Figure 4-2b).

Griffonia simplicifolia lectin I (GSL-I)

When one day old (Figure 4-1c), seven day old (Figure 4-2c) and 14 day old (Data not shown) kidneys were stained with GSL-I, it was found that in the cortex the lectin bound what appeared to be the capillary loop in the renal corpuscle, endothelial structures, and some tubules. Also, in some tubules in the medulla co-expression was found with Pax-2 and the lectin binding sites.

In renal papilla of one day old mice, the lectin stained the surface of most tubular structures containing Pax-2 positive cells (Figure 4-1c). However, later, in days 7 and 14, the lectin bound the surface of few tubules containing Pax-2 positive cells (Data not shown).

No GSL-I staining was observed in cortex or renal papilla of adult mice (Data not shown).

Solanum tuberosum lectin (STL)

When one day old kidneys were stained with STL lectin there was a weak lectin staining on the surface of the aggregates and S-shaped bodies in the nephrogenic zone (Figure 4-1c). STL lectin was also found to bind the surface of some tubular

structures in the cortex, and seemed to bind the renal corpuscle, but with lower intensity. In the renal medulla the lectin bound most of the tubules containing Pax-2 positive cells.

In day 7 (Figure 4-2c), day 14, and adult kidneys the lectin bound a few tubular structures in the cortex, but these tubules did not contained Pax-2 positive cells.

In renal papilla the lectin bound the surface of most of Pax-2 positive cells at all four time points (Figure 4-1c and Figure 4-2c).

4.2.2 Kidney stem cell isolation using magnetic-activated cell sorting (MACS®)

PNA was selected as the best lectin to enrich Pax-2 population in kidney using MACS. To determine whether PNA-coated magnetic beads could be used to isolate Pax-2 positive cells from mouse postnatal kidney, a single cell suspension of disaggregated kidney cells was incubated with PNA-coated beads, and the percentage of Pax-2 positive cells in the MACS-selected population was compared to that in the unselected population. Uncoated beads were included as controls.

No magnetic beads were observed in the unselected fractions obtained from PNA-coated and uncoated beads (Figure 4-5). Further analysis showed that while many of the cells in the unselected fraction obtained from control beads bound PNA, very few cells in the unselected fraction obtained from the PNA-coated beads bound PNA (Figure 4-5).

To determine the efficiency of the procedure, 24 hours following MACS, selected and unselected fractions from PNA-coated and uncoated beads were

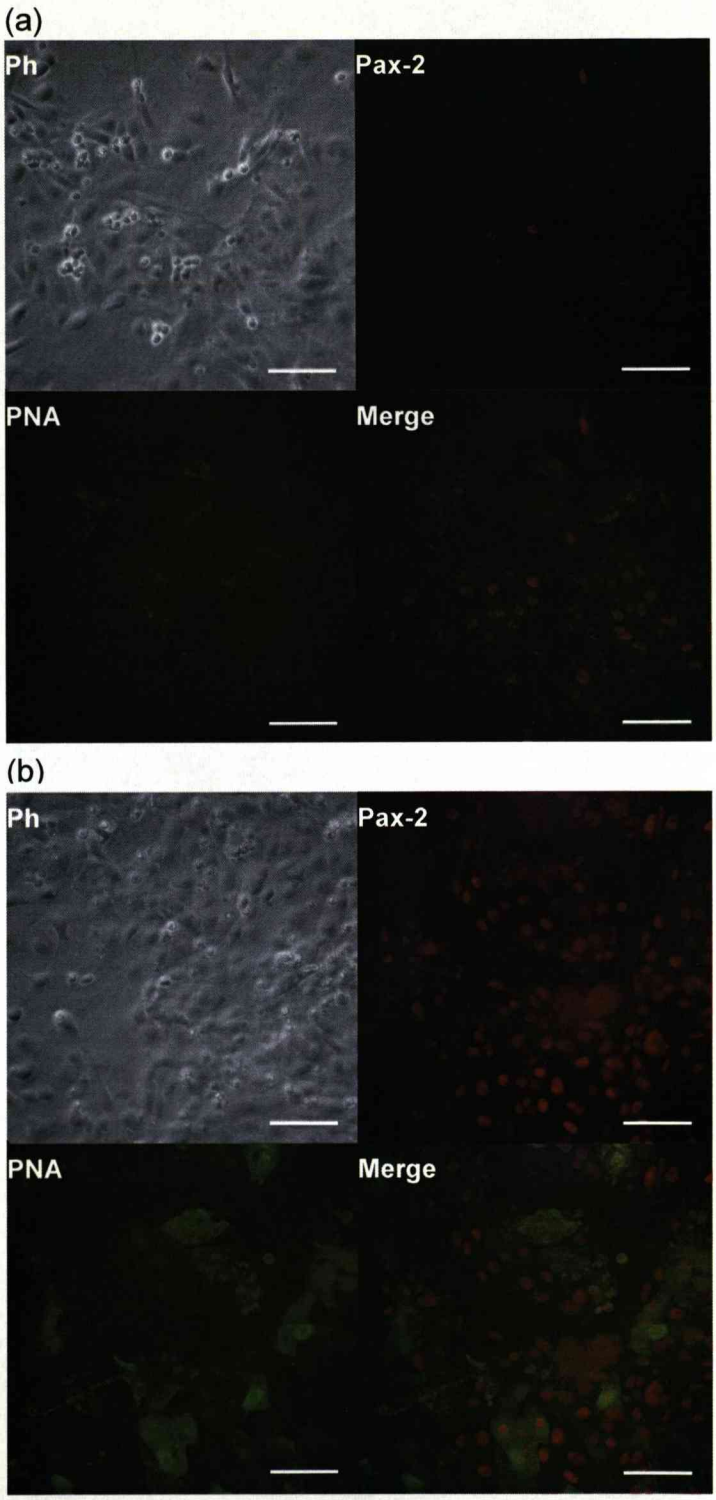


Figure 4-5 Micrographs showing the expression of Pax-2 (red) and lectin binding (green) in cells from the wash fractions from cells incubated with PNA-coated beads (a) and control beads (b). The cells from the control unselected fraction show significant levels of Pax-2 expression while those from the PNA selected fraction do not, indicating that the PNA-binding Pax-2 positive cells were retained in the column by the magnetic field. Ph – Phase contrast. Scale bars: 100 μm.

immunostained for Pax-2 to determine the proportion of Pax-2 positive cells in each fraction. The proportion of Pax-2 positive cells in the MACS-selected populations was then compared to that in a disaggregated kidney that had not been subjected to MACS.

It was found that 79% of cells selected with PNA-coated beads were Pax-2 positive, whereas only ~34% of cells in the unselected fraction were Pax-2positive (Figure 4-6). With the uncoated beads, ~30% of cells in the selected fraction were Pax-2 positive compared to ~65% in the unselected fraction.

Analysis of the disaggregated kidney not subjected to MACS, showed that following 24h in culture, 57% of the cells were Pax-2 positive.

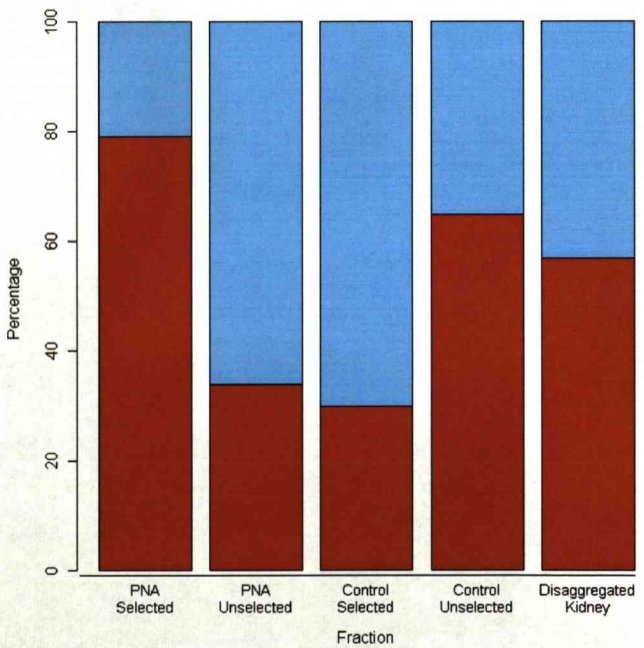


Figure 4-6 Proportions of cells from different cell fractions presenting Pax-2 expression after 24 hours in culture. The red portions of the bars represent the percentage of cells in the cultures that were Pax-2 positive.

Following plating of the MACS-selected cells, it was found that many of the cells were heavily loaded with beads (Figure 4-7). It has been shown previously that lectin binding can have effects on cellular processes such as proliferation, migration, and in some cases, can have a toxic effect on cells (Dodeur *et al.*, 1980; Ryder S., 1998; Schwarz *et al.*, 1999). Therefore, to avoid any potential problems arising from lectin binding to the kidney cells, attempts were made to remove the lectin-coated beads immediately after MACS selection by incubating

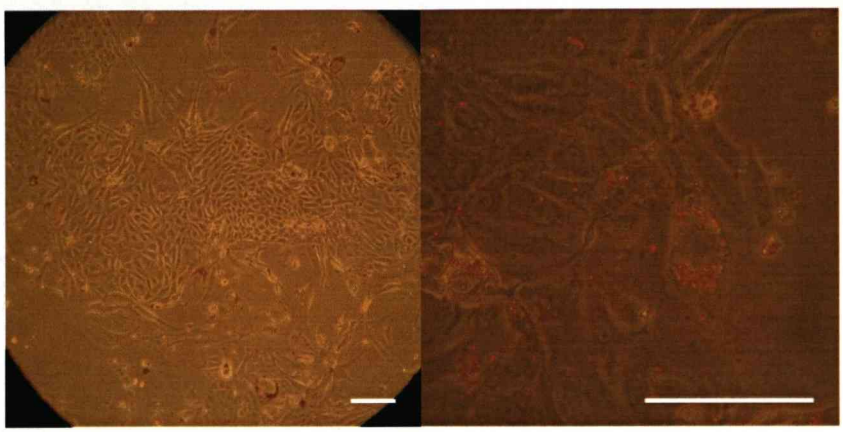


Figure 4-7 Micrographs of cell fraction selected with PNA coated beads. The left-hand image shows cells selected with PNA beads at 10× magnification. The right-hand side picture shows a higher magnification of cell in detail overloaded with magnetic beads. Scale bars: 100 μm.

the cells with the competing sugar, galactose. However, it was found that this strategy was not successful and the beads remained attached to the cells.

To determine whether the lectin-coated beads had any noticeable effect on cell viability, morphology or Pax-2 expression, the selected fractions of lectin-coated and uncoated beads, along with disaggregated kidney not subjected to MACS, were cultured for 2 weeks. After this period the cells were fixed and immunostained for Pax-2 (Figure 4-8).

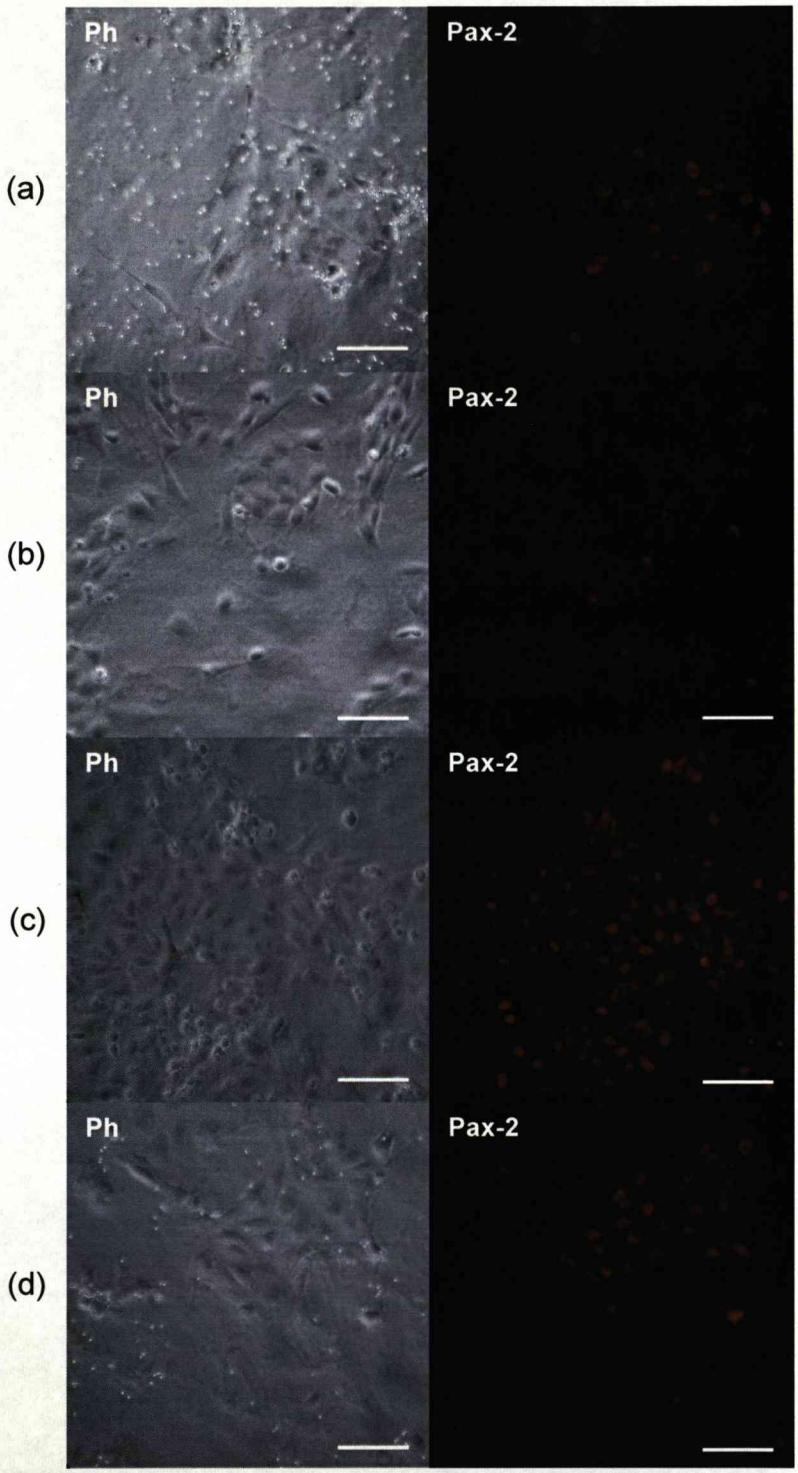


Figure 4-8 Micrographs showing cells from different cell fractions selected by MACS after 14 days in culture, and their Pax-2 expression. When cells separated with PNA beads (a & b) were cultured, the cells originating from the unselected fraction (b) showed depletion of Pax-2 positive cells while the selected fraction (a) showed enrichment of Pax-2 positive cells. When cells separated with control beads were cultured, the cells originating from the selected fraction (c) showed many cells expressing Pax-2 and cells from the unselected fraction (d) showed many Pax-2 positive cells as well. Ph – Phase contrast. Scale bars: 100 μm.

It was found that the PNA-selected cells and the cells from the disaggregated kidney showed little difference in cellular morphology or colony expansion. The cells selected with the unbound beads on the other hand displayed a lower number of colonies.

When the cells were studied for Pax-2 expression, colonies in which more than half of cells were Pax-2 positive were referred to as positive colonies, while colonies in which less than half were positive for Pax-2 were considered negative. Pax-2 analysis showed that in the cells selected with PNA-coated beads, 68% ofthe colonies were Pax-2 positive, while in those selected with uncoated beads only 14% of the colonies were Pax-2 positive. However, in the cells expanded from disaggregated kidney, 61% of the colonies were Pax-2 positive.

4.3 Discussion

In this chapter the isolation of Pax-2 positive cells from postnatal mouse kidney was attempted using MACS. In order to find a surface marker to isolate Pax-2 positive cells, a screening of lectins was performed in order to find a lectin that specifically bound the surface of the Pax-2 expressing cells in the papilla. It was found that high levels of the PNA lectin bound to the Pax-2 positive cells in the papilla, and PNA-coated magnetic beads could be used to enrich the Pax-2 positive population using MACS. However, it was found that even without MACS selection, the majority of isolated cells were Pax-2 positive following two weeks in culture.

Pax-2+ cells in the postnatal mouse kidney express binding sites for PNA lectin

PNA, which is specific for D-galactose or N-acetyl-D-galactosamine (Laitinen *et al.*, 1987; Young *et al.*, 1984), seemed to be the most suitable lectin among those tested for Pax-2 positive cell enrichment, as it bound the surface of most of the Pax-2 positive cells in the papilla at different stages of development. Hanai *et al.* (1994) showed that PNA bound proximal tubules in mouse kidney during development, from postnatal (including newborn, suckling, and weanling) to adult and senescent mice. Laitinen *et al.* (1987) also studied binding of PNA in mouse kidney and found that in late embryo and early neonates PNA bound the S-shaped body, the capillary loop, the proximal tubules, and the collecting ducts, and with less intensity, the primary cell condensates, primary vesicles, and distal tubules. Later on in development, when kidneys are matured, Laitinen *et al.* (1987) showed that PNA binding was restricted to proximal tubules and collecting ducts whereas Hanai *et al.* (1994) only found lectin binding to proximal tubular cells and not collecting ducts. The results of this study, which demonstrated that in early neonates strong lectin binding was observed in some tubular structures that may be proximal tubules and collecting ducts, are more consistent with the findings of Laitinen (Laitinen *et al.*, 1987). Weaker PNA binding was observed in other structures in the cortex such as early condensates and developing glomeruli.

In mature kidney, both groups found the lectin binding to be restricted to tubular structures, which is also consistent with the findings of this study that showed that after one week, PNA seemed to bind tubular structures in the cortex,

medulla, and papilla that may correspond to proximal tubules and collecting ducts.

However, in this study it was not possible to determine which type of tubules the lectin was binding to, and in order to determine this, immunostaining using specific tubular markers and the lectin would need to be done.

Enrichment of Pax-2+ cells using MACS

The magnetic separation using PNA coated beads seemed to enrich the population of Pax-2 expressing cells, however, a high percentage of Pax-2 cells was also obtained in whole plated kidney with no isolation procedure. The percentage of cells expressing Pax-2 was only 20% higher in the fraction of cells selected with PNA coated beads than in disaggregated kidney without isolation. Furthermore, after two weeks in culture, the percentage of colonies expressing Pax-2 was very similar in the cells selected with PNA beads and in the cells plated with no selection.

Following the hypothesis that Pax-2 positive cells may be a stem cell population, these cells may have a proliferation advantage *in vitro* compared with the rest of the cells. If this is the case, it may be possible that the percentage of Pax-2 positive cells present in plated kidneys with no isolation procedure is enough to proliferate and expand a stem cell population without MACS, or any particular cell separation procedure.

Magnetic cell isolation is an expensive technique and it is also time consuming, so overall it may be that isolation of cells using PNA coated beads is not the most

convenient method to isolate and enrich Pax-2 expressing cells. In previous attempts to isolate stem cells using MACS, cells have been isolated using particular surface markers that are expressed by the cell population of interest, such as CD133 (Bussolati *et al.*, 2005; Sagrinati *et al.*, 2006). The lack of a cell surface marker to isolate Pax-2 expressing cells makes the isolation difficult. The use of PNA binding to isolate the cells as opposed to the actual marker, Pax-2, for the cells of interest reduced the efficiency of the procedure, given the fact that PNA was not fully specific for Pax-2.

Another approach could have been to isolate cells not expressing Pax-2 in order to deplete Pax-2 negative cells from the total population and leave Pax-2 positive cells in the wash fraction. A third possibility could have been the combination of positive and negative selection using PNA and PHA-L to further enrich population of Pax-2 positive cells. PHA-L lectin seemed to be ideal for negative selection. PHA-L showed a constant pattern during development of not binding Pax-2 positive cells.

In conclusion, although the MACS technique did isolate high numbers of Pax-2 positive cells from kidney, it appeared that plating the cells from disaggregated kidney was equally effective for obtaining high numbers of Pax-2 expressing cells *in vitro*. After six weeks in culture there was no observable difference in the numbers of Pax-2 positive colonies in cultures from the two techniques, therefore, it was decided that the simpler option of using whole kidney disaggregates was a more efficient means of proceeding. However, further optimisation of the culture conditions might offer the possibility of encouraging greater proliferation of the

Pax-2 positive cells relative to the other cells in the kidney should they prove to be stem cells. In the next chapter, culture conditions to expand Pax-2 positive cells from disaggregated kidney were optimized.

Chapter 5: *In vitro* expansion of putative stem cells

5.1 Introduction

In order to work with stem cells it is necessary to have the correct conditions to culture them for two different cellular states. First, stem cells need to be maintained in a self-renewing state of undifferentiating proliferation. Second, when it is required, stem cells need to be differentiated under the right stimulus to a particular cell type. A crucial factor is the substrate on which the cells are grown, which influences proliferation, differentiation, and phenotype of the cells.

Mouse embryonic stem cells can be maintained undifferentiated *in vitro* in the presence of a feeder layer of fibroblasts (Evans and Kaufman, 1981a; Evans and Kaufman, 1981b; Martin, 1981) or, alternatively, on gelatin-coated plates with the addition of leukemia inhibitory factor (LIF) (Williams *et al.*, 1988). The fibroblast feeders provide not only a substrate on which to grow the stem cells, but also supply the stem cells with growth factors essential for survival (Smith and Hooper, 1983; Talbot *et al.*, 1994). Although it is possible to maintain the cells undifferentiated with no feeders and using media supplemented with LIF, most groups still culture the cells on feeders to provide the necessary factors to promote proliferation of the cells in an undifferentiated state.

The most common types of fibroblast used as feeder layers to grow embryonic stem cells are mouse embryo fibroblasts (MEFs) and STO mouse fibroblast cell line (STO). STO cells are thioguanine- and ouabain-resistant subline of SIM mouse fibroblast isolated by Dr. A. Bernstein (Martin and Evans, 1975). STO are

an established cell line so they can be grown in the lab easily. MEFs, by contrast, need to be continuously replenished from frozen stocks.

Several problems can arise from the use of feeders. A major problem of using feeders to grow stem cells arises when analysing the cells. Given the cellular nature of the feeders, they can interfere with the results by giving background staining and signals in immunostainings and polymerase chain reaction (PCR) analyses. In addition, it is impossible to know the influence that feeders have on the stem cells behaviour.

When fibroblasts are grown *in vitro* they produce a pericellular matrix that contains fibronectin (Hedman *et al.*, 1978; Hedman *et al.*, 1979), procollagen types I and III and glycosaminoglycans (Hedman *et al.*, 1979). This pericellular matrix can be isolated by removing the fibroblasts using a 0.5% sodium deoxycholate solution leaving a substrate suitable for culture without the presence of unwanted cells (Hedman *et al.*, 1979). This could provide a solution to the problems associated with the presence of the fibroblasts themselves. However, it would then be necessary to replace the factors that feeders release into the medium. Some groups have used conditioned media from STO cells in order to supplement stem cells with factors normally released by the feeders (Ouyang *et al.*, 2007).

Another approach to growing kidney cells is to coat the dishes with extracellular matrix (ECM) components in order to mimic the *in vivo* process. Different components of the ECM have been used to grow and expand KSC such as Matrigel™, Type I, and Type IV collagen, laminin and fibronectin (Draper *et al.*,

2004; Maeshima *et al.*, 2006). Fibronectin, which is an adhesive ECM protein, seems to be the ECM component of choice for KSC expansion (Bussolati *et al.*, 2005; Gupta *et al.*, 2006; Oliver *et al.*, 2004; Sagrinati *et al.*, 2006). Fibronectin-rich matrices support and influence cell proliferation, survival, migration, and differentiation (George *et al.*, 1993; McCarthy *et al.*, 2003). ECM plays an important role in kidney development and also repair, and is involved in processes such as ureteric bud branching morphogenesis and metanephric mesenchyme condensation. It also has an influence on the differentiation of the renal tubes, and the assembly of the glomerular basement membrane (Lelongt and Ronco, 2003).

The most frequently used media for culturing stem cells is Dulbecco's modified Eagle's medium (DMEM) containing 4500 mg/l of glucose with the addition of 1 mM sodium pyruvate and a high concentration of serum (10-20%) (Hogan *et al.*, 1994).

In order to reduce the concentration of serum added to the culture media there are some commercially available media that allow reduction of serum without resulting in a changing in the morphology of the cells or cell growth rate. For example, Advanced DMEM has been used by various groups in to promote growth and proliferation of stem cells in a low serum concentration (Boucher *et al.*, 2006).

It has been also shown by Dudley *et al.* (1999) that the addition of FGF-2 and BMP-7 to the culture media promote survival and proliferation of metanephric mesenchyme cells. Potentially the addition of these two factors to the medium

could increase the chances of survival and proliferation of KSC or metanephric mesenchyme-like cells.

5.1.1 Aims

The aim of this chapter was to test different substrates and cell culture media for their suitability in culturing the isolated population of putative kidney stem cells (pKSC). The culture dishes were coated with different substrates, and each substrate was assessed for its ability to promote attachment, proliferation and expansion of pKSC, while maintaining the "undifferentiated" state of the pKSC characterised by the expression metanephric mesenchyme markers.

5.2 Results

5.2.1 Establishing the optimal culture medium for expansion of putative KSC

To test which culture medium was most suitable for expansion of the KSC, the cells were plated on dishes coated with fibronectin with two different media: Advance DMEM with no added serum and STO-conditioned medium with 2.5% FCS added (STO-CM). When the cells were grown on Advanced DMEM with no added serum, the cells attached to the dish and proliferated. After a week to ten days the proliferation slowed down and it was difficult to subculture them successfully (Figure 5-1). When the cells plated on fibronectin coated dishes were grown on STO-CM the cells attached and proliferated continuously, and it was possible to subculture the cells every few days. Cells in these conditions could be maintained long term in culture, hence STO-CM was selected as the basic media for further optimisations.

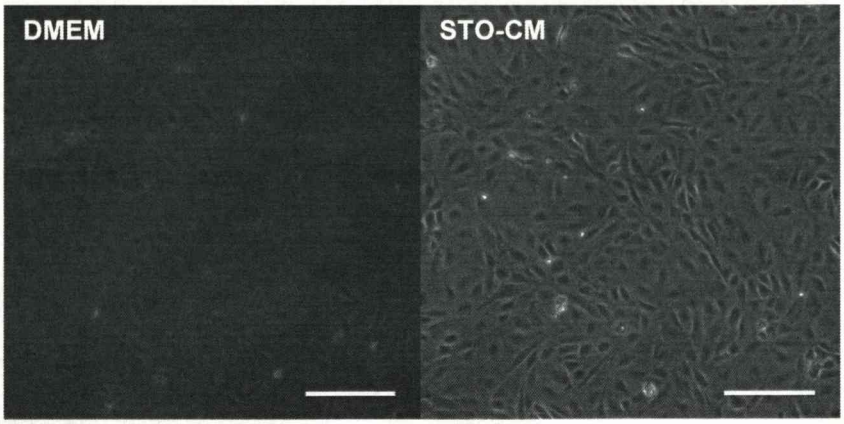


Figure 5-1 Phase contrast micrograph showing cells isolated from 3 days old mouse kidney after 10 days in culture. Cells were plated on fibronectin coated dishes with two different media – Advanced DMEM with no serum and STO-CM. Scale bars:200μm.

BMP-7 and FGF-2 in combination have been shown to encourage the growth and maintenance of MM cells *in vitro* (Dudley *et al.*, 1999). In order to evaluate their effects on expansion and proliferation of the pKSC, the cells were cultured on substrates of fibronectin or dSTO with one of two different media. The first was STO-CM and the second was STO-CM to which BMP-7 and FGF-2 had been added.

The cells were cultured under each of the four different conditions for two weeks. No differences were observed in the morphology or the growth rate of the cells between the four conditions (Figure 5-2). After two weeks in culture, cells were collected from each condition and RNA was extracted for RT-PCR analysis of MM markers.

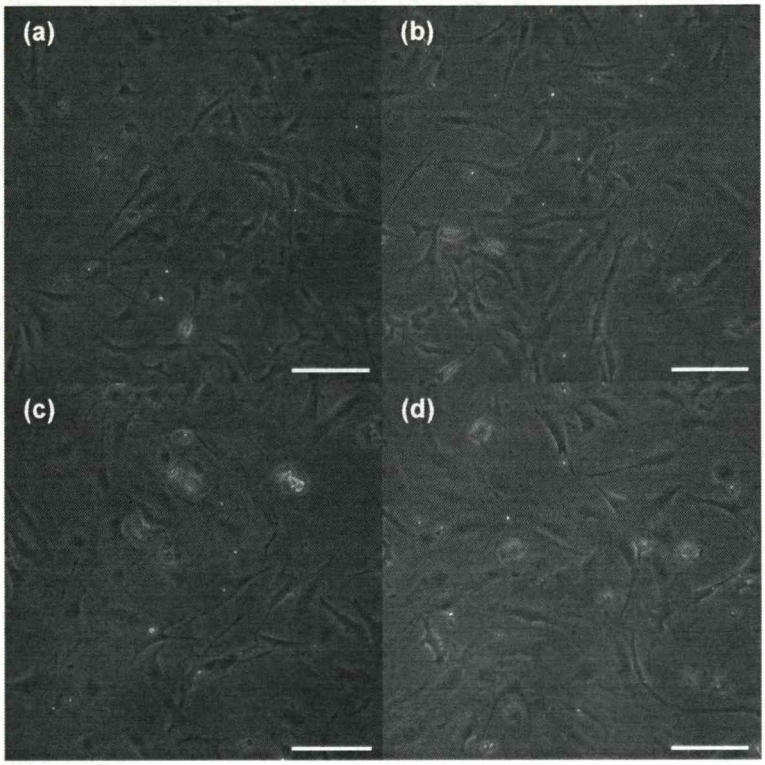


Figure 5-2 Phase contrast images of cells grown on dSTO and fibronectin coated dishes for 5 days with without or with added growth factors. (a) Cells grown on dSTO for 5 days with STO-CM. (b) Cells grown on dSTO for 5 days with STO-CM plus FGF-2 and BMP-7. (c) Cells grown on fibronectin coated dishes for 5 days with STO-CM. (d) Cells grown on fibronectin coated dishes for 5 days with STO-CM plus FGF-2 and BMP-7. Scale bars: 100 μm.

A band of the specific size of Pax-2 (303 bp) was observed in the RNA extracted from a whole 5 day old mouse kidney; this was used as a positive control for Pax-2. No Pax-2 band appeared on heart and liver of five days old mouse; this was used as negative controls for Pax-2 expression. Pax-2 was expressed by the cells grown in all four conditions. There was no obvious difference in the intensity of the Pax-2 bands between the four conditions. No band for Pax-2 was observed on the reaction where no template was added. The results of RT-PCR for Pax-2, WT1 and GDNF are shown in Figure 5-3.

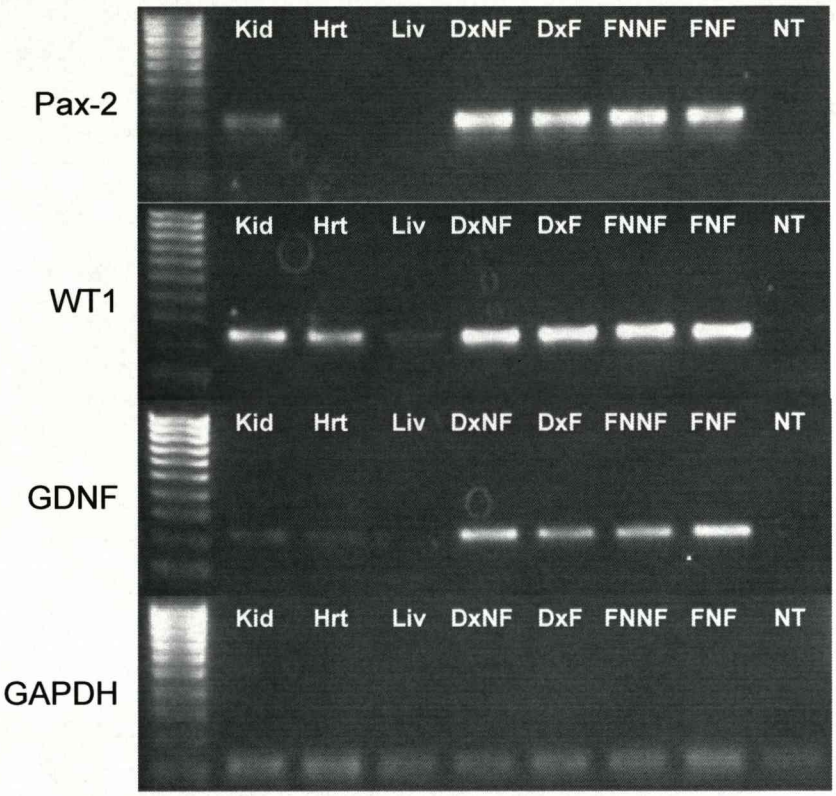


Figure 5-3 Reverse transcription-polymerase chain reaction for Pax-2, WT1, GDNF and GAPDH from RNA extracted from cultured kidney cells with whole kidney, heart and liver from 5 day old mouse as controls. Kid – whole kidney; Hrt – whole heart; Liv – liver; DxNF – cells grown on dSTO with no added factors; DxF – cells grown on dSTO with FGF-2 and BMP-7 added to the medium; FNNF – cells grown on fibronectin with no factors added; FNF – cells grown on fibronectin with FGF-2 and BMP-7 added to the media; NT – no template control.

A band corresponding to WT1 (234 bp) was observed on the whole 5 days old mouse kidney and also another band on the whole heart from five days old mouse; both bands presented similar intensity. A weaker band appeared for WT1 expression on the five day old liver sample. Cells from all four conditions presented a band of similar intensity corresponding to WT1 expression. No band was observed when no template was added.

The next marker analyzed was GDNF (176 bp). Weak bands were observed on the samples from five days old heart and kidney. No band for GDNF was observed on the five days old neonate liver. GDNF was expressed on cells grown

on dSTO and on fibronectin with or without added factors, all four bands had similar intensity. No GDNF band was observed on the reaction with no added template.

The reference gene GAPDH (110bp) showed a similar level of expression in all the samples.

5.2.2 Establishing the optimal culture substrate for expansion of putative KSC

To determine the optimal culture conditions for expansion of putative KSC, disaggregated cells from 3 days old neonatal mouse kidneys were plated onto the following four culture substrate: Matrigel, STO feeders, deoxycholate-treated STO feeders (dSTO) and fibronectin. Matrigel did not support the growth and expansion of the renal cells so it was discard in early experiments (Data not shown).

5.2.3 Short term culture of renal cells on three substrates

As a preliminary test to investigate whether the plated cells from neonatal kidneys had a KSC-like phenotype after short term culture, the cells were fixed and stained for Pax-2 on the 1st and 7th day after plating.

After one day in culture, the cells plated onto STO feeders had formed many colonies of different sizes, and most of the cells in these colonies were Pax-2 positive (Figure 5-4 a). The colonies were not very large, with between 80-90%

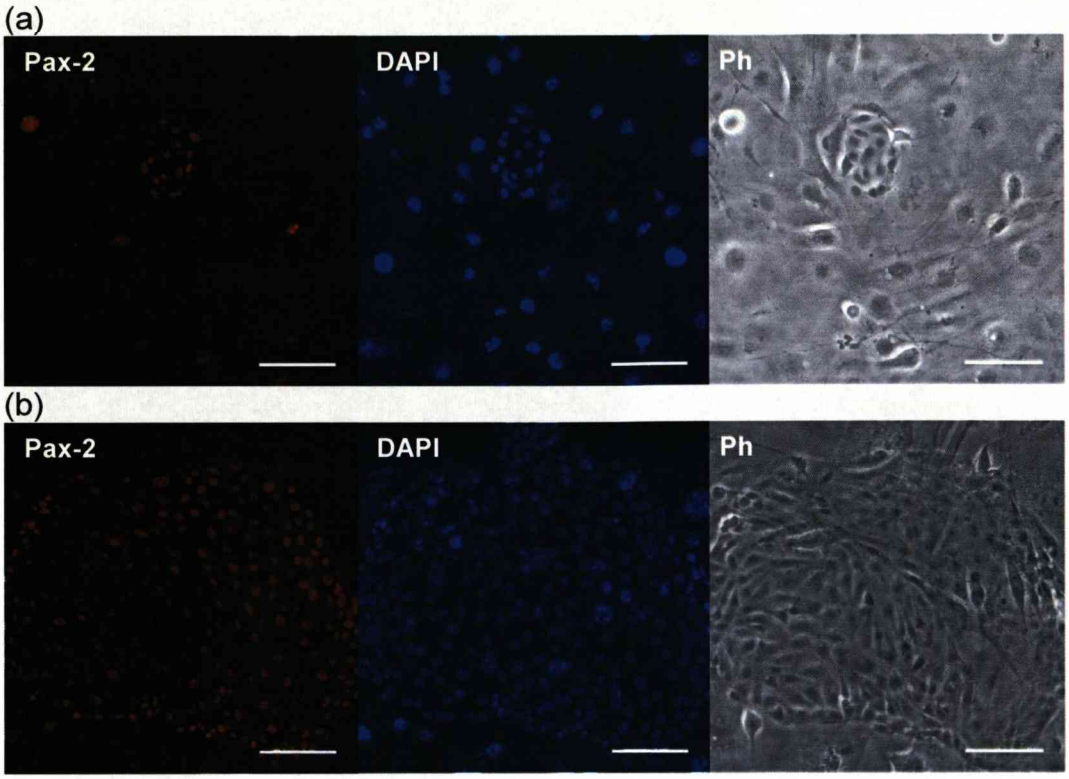


Figure 5-4 Micrographs showing immunostaining for Pax-2 and DAPI, and phase contrast of cells from whole disaggregated kidney of 3 days old neonatal mice plated on STO feeder cells. (a) Cells grown on STO feeders and fixed after one day in culture stained. (b) Cells grown on STO feeders and fixed after one week in culture. Ph – phase contrast. Scale bars: 100 μm.

of the colonies containing less than 15 cells. After one week, the colonies appeared considerably bigger and still expressed Pax-2 (Figure 5-4 b). The cells grew as a monolayer and presented mainly an epithelial morphology. Approximately half of the colonies now contained more than 15 cells.

When the renal cells were plated onto dishes coated with deoxycholate-treated STO (dSTO), after one day in culture the cells were found to attach to the dish forming a monolayer of colonies of different sizes (Figure 5-5 a). Again the colonies were relatively small, with about 80-90% of them containing fewer than 15 cells. Some single cells were found between the colonies or close to the periphery of the colonies. Cells of different morphology were observed, with

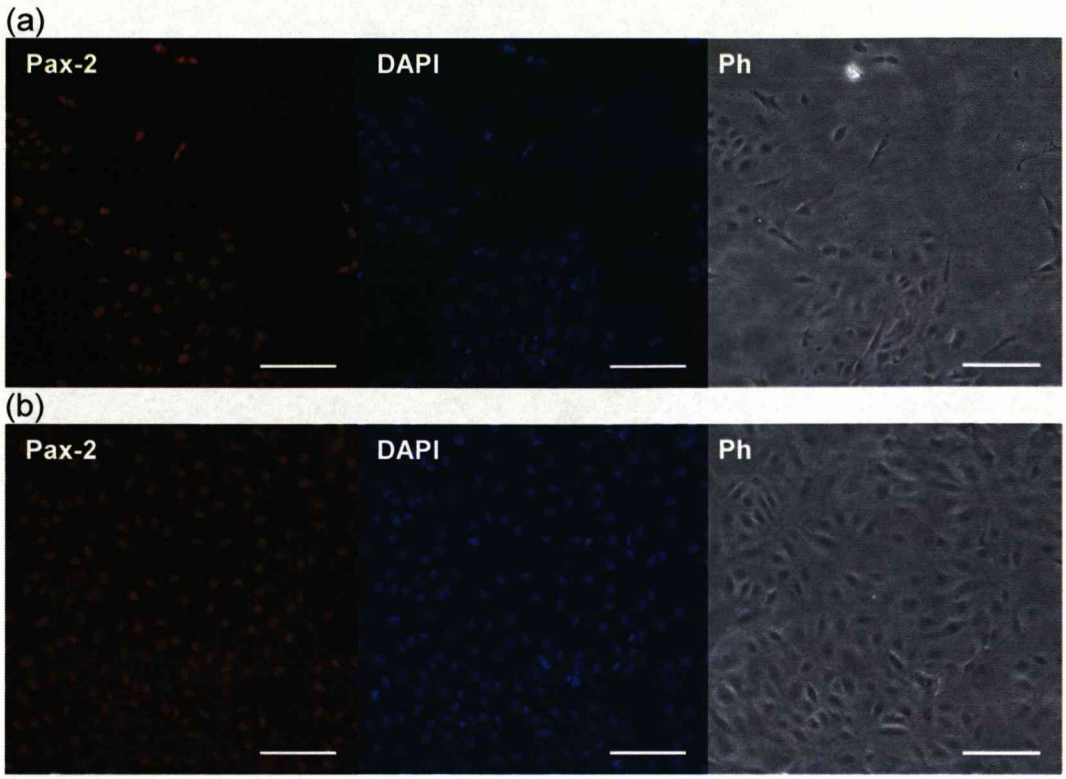


Figure 5-5 Micrographs showing immunostaining for Pax-2 and DAPI, with phase contrast of cells from whole disaggregated kidney of 3 days old neonatal mice plated on dSTO feeder cells. (a) Cells grown on dSTO feeders and fixed after one day in culture. (b) Cells grown on dSTO feeders and fixed after one week in culture. Ph – phase contrast. Scale bars: 100 μm.

some of the cells being elongated whilst others were more rounded. Some of the cells seemed to form epithelial-like colonies of thin flat layers. When the cells were left for a week under this condition they proliferated and spread over the surface of the dish, coating most of it (Figure 5-5 b). Most of the cells still expressed Pax-2.

Cells were plated onto fibronectin coated dishes expressed Pax-2 after one day in culture. At this point colonies of different sizes were found and a similar morphology was observed if they were compared with the cells plated on feeders and dSTO (Figure 5-6 a). It was also possible to observe some single cells attached to the dish very close to the colonies or on the free space between the

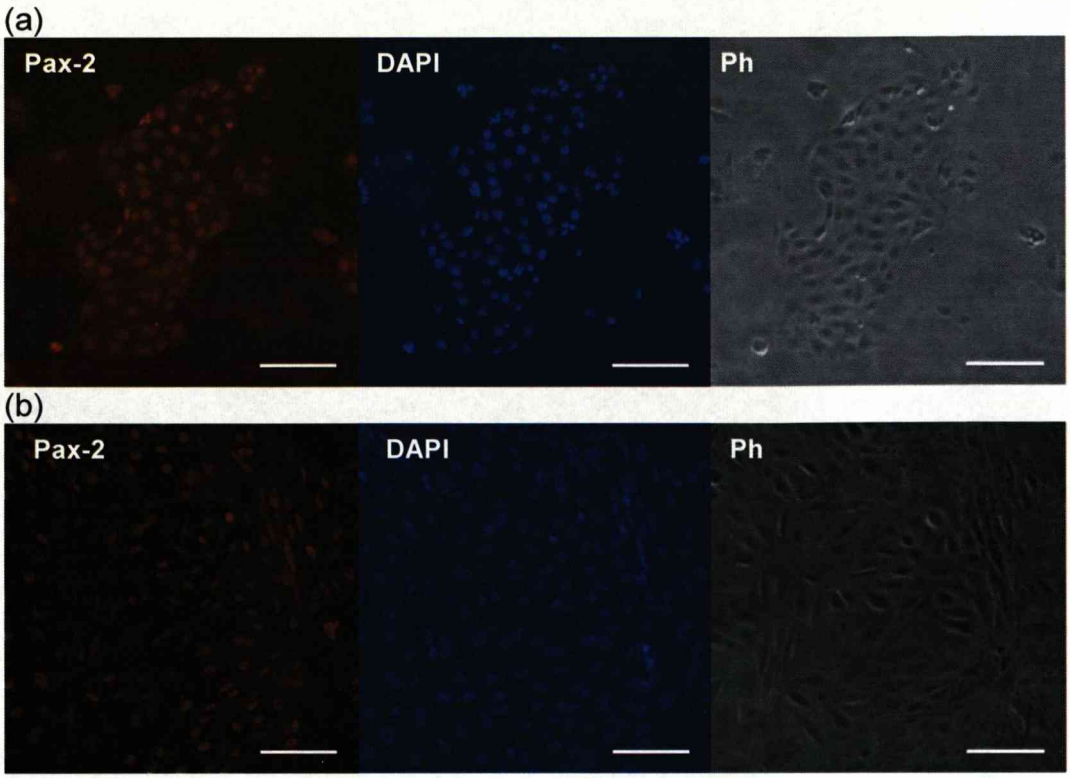


Figure 5-6 Micrographs showing immunostaining forPax-2 and DAPI, with phase contrast, of cells from whole disaggregated kidney of 3 days old neonatal mice plated on fibronectin coated dishes. (a) Cells grown on fibronectin and fixed after one day in cultura. (b) Cells grown on fibronectin and fixed after one week in cultura. Ph- phase contrast. Scale bars: 100 μm.

colonies. In this condition, as the previous two, 80-90% of the colonies were smaller than 15 cells. When the cells were left for a week on fibronectin coated dishes, it was possible to observe proliferation, and the cells were found to spread along the surface of the dish, coating most of it after one week (Figure 5-6 b). Nearly all still maintained Pax-2 expression. Some cells formed epithelial-like colonies, whilst other cells, with more elongated morphology, covered the rest of the dish and surrounded these colonies.

5.2.4 Long term culture of renal cells on three substrates

Cells grown for one month

The cells were grown for a month on STO feeders and were checked for Pax-2 expression (Figure 5-7). After this time many cells still expressed Pax-2, but some cells started to lose Pax-2 expression. In some colonies pairs of cells were observed with closely adjacent nuclei, suggestive of daughter cells that had arisen from a recent cell division. In many cases, one daughter was Pax-2 positive and the other Pax-2 negative. It was found that after a few passages the cells on feeder layers did not expand any further, and that they could not be cultured for more than 1 month.

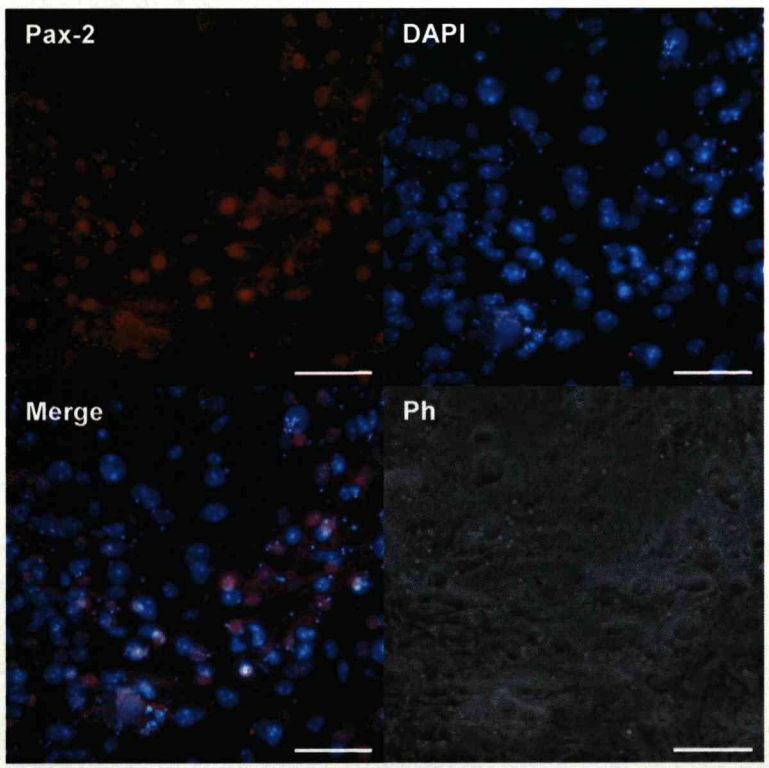


Figure 5-7 Micrographs showing immunostaining for Pax-2 and DAPI, merge of both markers, and phase contrast of cells from whole disaggregated kidney of 3 days old neonatal mice plated onto STO feeder cells and cultured for one month. Ph – phase contrast. Scale bars: 100 μm.

Cells grown on dSTO and on fibronectin were also grown for a month in culture, however, no changes were observed in these two conditions and the expression of Pax-2 and WT1 was maintained, therefore the cells were cultured for a further 4 months.

Cells grown for four and eleven passages

At this point, STO feeders were discarded as a substrate as they did not support the expansion of renal cells and resulted in the loss of the expression of Pax-2 in most of the surviving cells.

Cells grown for four and eleven passages (approximately two and five months in culture respectively) on deoxycholate treated STO and on fibronectin were fixed and immunostained for Pax-2 and WT1. When the cells grown for four passages on dSTO were analysed, the majority of the cells were found to express Pax-2 and WT1 (Figure 5-8). Among the cells expressing Pax-2 and WT1 different levels of expression were observed. When the cells were grown on dSTO for eleven passages there was a decrease in the number of cells expressing Pax-2, however, WT1 was still expressed in most of the cells (Figure 5-8).

When the cells were grown for four passages on fibronectin coated dishes, a similar staining pattern to the cells grown on dSTO was observed (Figure 5-9). Most of the cells were positive for Pax-2 and also WT1. The cells grown on fibronectin for eleven passages also presented similar staining than cells grown on fibronectin for four passages with most of the cells Pax-2 positive and also WT1 positive (Figure 5-9).

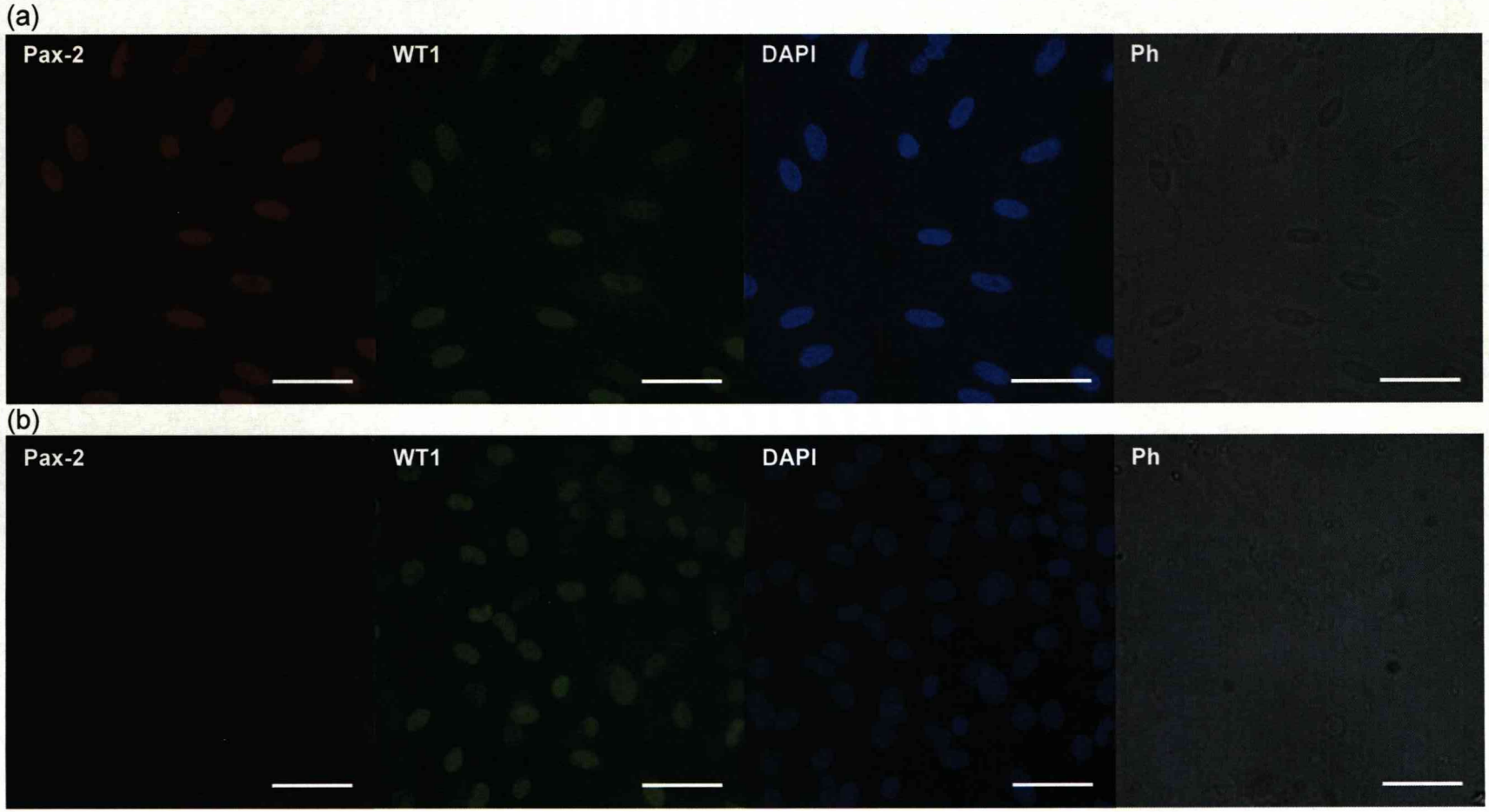


Figure 5-8 Micrographs showing immunofluoresce for Pax-2, WT1, and DAPI, with phase contrast images of KSC cells grown on deoxycholated treated STO dishes. (a) KSC grow on dSTO for four passages. (b) KSC grown on dSTO for eleven passages. Scale bars: 50μm.

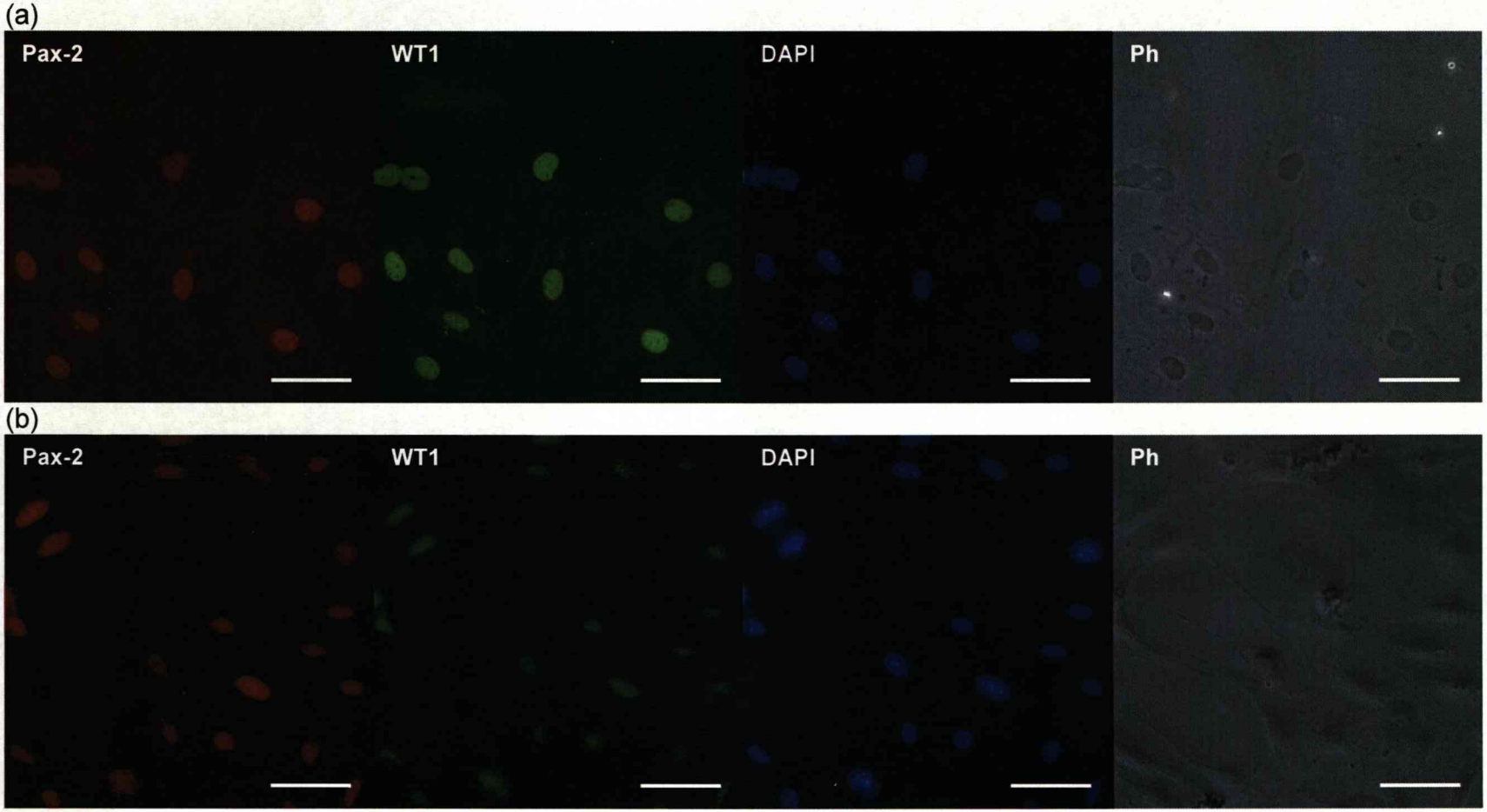


Figure 5-9 Micrographs showing immunofluoresce for Pax-2, WT1, and DAPI, with phase contrast images of KSC cells grown on fibronectin coated dishes. (a) KSC grow on fibronectin for four passages. (b) KSC grown on fibronectin for eleven passages. Scale bars: 50μm.

The cells grown on dSTO and fibronectin for four and eleven passages were also analysed by RT-PCR (Figure 5-10). At passage four, the cells presented a specific band with similar intensity for Pax-2 expression on both conditions. Pax-2 bands were also observed on the controls: whole kidney rudiments of E11.5 embryos and whole kidney of five day old neonate. The cells grown on both substrates presented WT1 expression at passage four with similar levels. All the controls presented some level of WT1 expression, stronger in the case of neonatal kidney and heart and slightly weaker on the E11.5 whole rudiment. An even weaker expression level was observed on the neonatal liver and on the STO feeder cells.

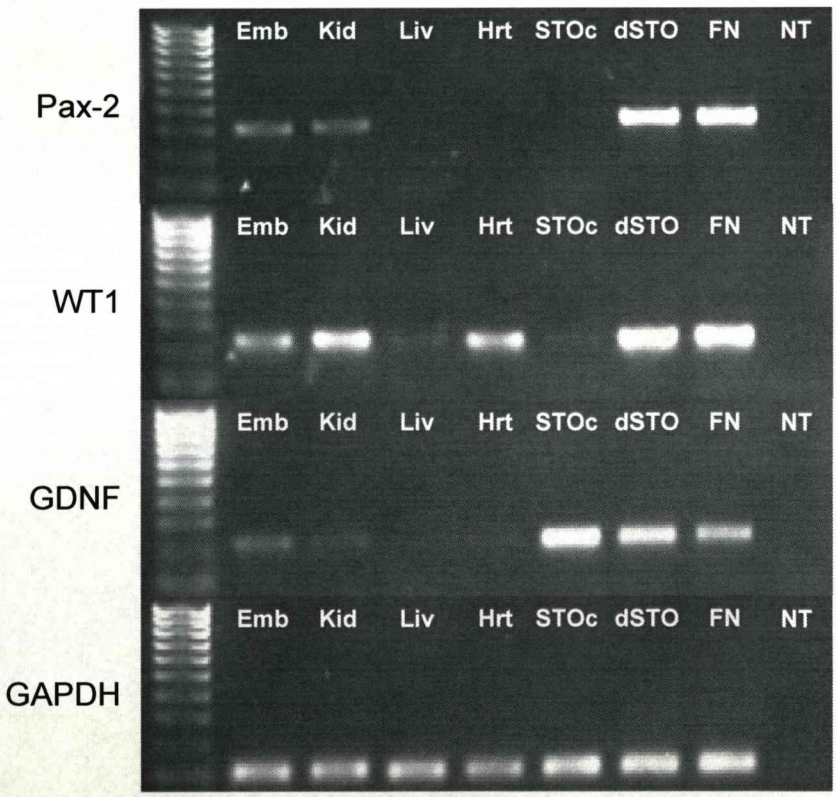


Figure 5-10 Reverse transcription-polymerase chain reaction for Pax-2, WT1, GDNF and GAPDH from RNA extracted from cells cultured for four passages on dSTO and fibronectin, with STO feeder cells, kidney rudiments, whole kidney, liver and heart of 5 day old mouse as controls. Emb – E11.5 kidney rudiment; STOc – STO feeder cells; Kid – whole kidney; Liv – liver; Hrt – whole heart; FN – fibronectin; NT – no template control.

A band appeared confirming the presence of GDNF at passage four on the cells grown on both substrates. Also the STO cells presented a clear band of GDNF expression, and weaker bands were observed in whole kidney rudiments of E11.5 embryos and whole kidney of five day old neonate. A weak band appeared in the heart sample.

The next time point analysed was passage eleven (Figure 5-11). Pax-2 was expressed in the kidney of five day old neonate, which was used as a positive control. There was a very weak band of Pax-2 in the STO cells. There was no Pax-2 expression in the heart. There was Pax-2 expression in the cells plated on deoxycholate treated STO, but the expression seemed weaker than in passage four.

Pax-2 expression was maintained at passage eleven in cells grown on fibronectin, and Pax-2 levels were clearly lower on the cells grown on dSTO than in cells grown on fibronectin (Figure 5-11). WT1 was expressed in kidney and heart of five day old neonate, and by STO feeder cells. Similar levels of WT1 expression were found on cells grown on both substrates, and the levels appeared to be maintained from passage four. GDNF was found to be expressed in the kidney of five day old neonate, STO feeder cells, and very weakly in heart. GDNF was also expressed in the cells grown on both substrates although the levels seemed to be higher on the cells grown on fibronectin. Sall1 was not expressed by any of the controls or the cells grown on dSTO, but was expressed in the cells that were grown on fibronectin for eleven passages. Similar levels of expression of the reference gene, GAPDH, were found in all samples.

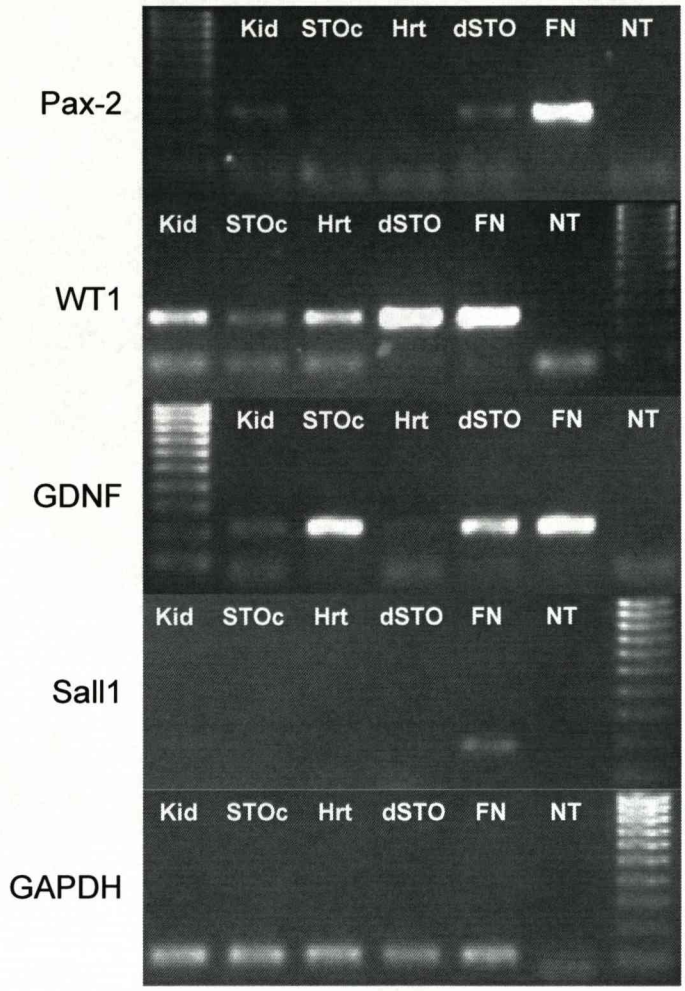


Figure 5-11 Reverse transcription-polymerase chain reaction for Pax-2, WT1, GDNF, Sall1, and GAPDH from RNA extracted from cells cultured for eleven passages on dSTO and fibronectin and also whole kidney, STO feeder cells, and heart of 5 dayl old mouse as controls. Kid – whole kidney; STOc – STO feeder cells; Hrt – whole heart; FN – fibronectin; NT – no template control.

5.3 Discussion

In this chapter, culture conditions were optimized for expanding the Pax-2 positive cells isolated from disaggregated kidney. By culturing on a substrate of fibronectin in medium conditioned by the STO mouse embryonic fibroblast cell line, it was found that the cells could be expanded, and could maintain expression of metanephric mesenchyme markers.

STO-conditioned medium (STO-CM) supports expansion of Pax-2+ cells

Cells grown in conditioned medium fared better than cells in Advanced DMEM medium without serum. This could be due to the factors present in the medium that were previously released by the STO feeders, and that provided the cells with the necessary factors to proliferate. For instance, it was found that the STO cells expressed high levels of GDNF, which apart from playing an important role in kidney development, has been shown to promote the proliferation and survival of several types of stem and progenitor cell types, including spermatogonial stem cells, enteric neuron and glial progenitors (He *et al.*, 2008; Heuckeroth *et al.*, 1998).

However, it is also possible that the FCS (2.5 %) contained in the STO-CM was responsible for the improvement in survival and proliferation of the cells grown in this condition.

The addition of the growth factors BMP-7 and FGF-2 to the STO-CM did not affect the expression of the MM markers Pax-2, WT1, or GDNF, nor did it improve proliferation or expansion of the cells. This is in contrast to the improvement in survival and proliferation of MM cells reported by Dudley *et al.* (1999). Dudley *et al.* (1999) used MM cells in their study, while the cells used in this study were derived from neonatal kidneys. It could be that the cells used in this study were originally derived from MM, however, they may have a different level of renal commitment or differentiation and this may explain their lack of response to BMP-7 and FGF-2.

Fibronectin culture substrate supports expansion of Pax-2+ cells

The kidney cells plated on STO feeders, dSTO, or fibronectin were isolated from disaggregated neonatal mouse kidney (three to five days old). It was shown in chapter 3 that at this neonatal stage, only two areas in the kidney contained a high number of Pax-2 positive cells, the nephrogenic zone in the cortex and the papilla. It is therefore surprising that following two weeks of *in vitro* culture, most of the cells on all three culture substrates expressed Pax-2, considering that they were isolated from whole kidney. One possible explanation could be that Pax-2 positive cells have an advantage compared with Pax-2 negative cells. They may have a better capacity to attach to the dishes and to survive in culture than cells not expressing Pax-2. Another explanation could be that the *in vitro* conditions change the cell phenotype and induce them to express Pax-2. However, further experiments would need to be done to determine which was the case.

Following long term culture, it was found that STO cells and dSTO did not support the expansion of kidney cells expressing MM markers such as Pax-2.

Many adjacent cells with close nuclei, probably derived from a recent cell division, were observed among the cells growing on STO. Interestingly, in some cases one daughter cell was Pax-2 positive and the other had lost Pax-2 expression. This phenomenon could be due to the cells undergoing asymmetric division. Asymmetric division is a frequent feature of stem cells that allows them to maintain their numbers (self-renew) whilst also generating progenitor cells or more differentiated cells (Morrison and Kimble, 2006). This suggests that the

cells maintained long-term on STO feeders started to undergo a differentiation process and were differentiating to a more mature phenotype.

Pax-2 and WT1 are co-expressed in MM in early kidney development. During kidney development Pax-2 is expressed in condensing mesenchyme and comma-shaped bodies, but starts to decrease in the proximal part of the S-shaped bodies as WT1 expression begins to increase (Ryan *et al.*, 1995). It could be that the cells cultured on STO and dSTO for longer periods were starting to differentiate and to acquire a more mature renal phenotype and therefore, started to lose Pax-2 expression.

It appears that kidney cells plated on fibronectin coated dishes and in STO conditioned medium were best able to proliferate and expand in culture in the long term whilst maintaining expression of MM markers, such as Pax-2, WT1, GDNF, and Sall1.

In conclusion, in this chapter the conditions for expanding the Pax-2 cell population *in vitro* were optimized. Based on their expression of the metanephric mesenchyme markers Pax-2, WT1, Sall1, and GDNF it was hypothesised that these cells might represent a population of stem cells. In the next chapter these MM-like cells were further characterized in order to determine if these renal cells were kidney stem cells.

Chapter 6: Characterization and differentiation of expanded KSC

6.1 Introduction

The previous chapters described the identification, isolation and culture of a putative stem cell population isolated from neonatal mouse kidney. These cells were found to express a number of MM-specific genes, raising the possibility that they could be MM-like cells that remain in the kidney following nephrogenesis. In order to establish whether these cells were stem cells, it was necessary to determine their characteristics. It is well established that the defining property of a stem cell is that it should be able to self-renew, giving rise to more stem cells, and differentiate into specific cell types (Morrison and Kimble, 2006). Therefore, stem cells can be identified on the basis of their properties of self-renewal, clonogenicity, and also differentiation potential and/or expression of specific markers (Sagrinati *et al.*, 2006).

In this chapter putative kidney stem cells were differentiated to specific renal and non-renal cells.

Stem cells have been shown to be present in most adult tissues, such as skin (Gambardella and Barrandon, 2003; Tumbar *et al.*, 2004; Watt and Hogan, 2000), intestinal epithelium and bone marrow (Moore and Lemischka, 2006; Pittenger *et al.*, 1999), central nervous system (Reynolds and Weiss, 1992), retina (Tropepe *et al.*, 2000), skeletal muscle (Jackson *et al.*, 1999), liver (Alison and Sarraf, 1998), and kidney (Bussolati *et al.*, 2005; Challen *et al.*, 2006; Gupta *et al.*, 2006;

Hishikawa *et al.*, 2005; Iwatani *et al.*, 2004; Kitamura *et al.*, 2005; Maeshima *et al.*, 2006; Oliver *et al.*, 2004; Sagrinati *et al.*, 2006).

Tissue-specific stem cells were originally considered to be capable of only giving rise to the cell types that form their tissue of origin, however, it has been proved that at least some adult stem cells, such as mesenchymal stem cells, can generate cell types from a variety of tissues, such as bone, cartilage and fat (Herzog *et al.*, 2003; Jiang *et al.*, 2002).

This has also been shown to be the case with kidney stem cells. Sagrinati *et al.* (2006) isolated a population of progenitor cells from human kidney and were able to generate not only renal cell types, but also extrarenal cell types such as osteoblasts and adipocytes.

The nephron is the structural and functional unit of the kidney. Each nephron is formed by the renal corpuscle (Bowman's capsule and glomerulus) and the renal tubule. The Bowman's capsule is the initial portion of the nephron and it is invaded by the vascular tuft, which is the glomerulus. The renal corpuscle contains the filtration apparatus of the kidney, which is comprises three components: the vascular endothelial cell layer, an outer epithelial cell layer that contains specialised cells called podocytes, and an intermediate layer of basement membrane called glomerular basement membrane (GBM). (Vize *et al.*, 2003).

Podocytes stabilize glomerular architecture and form a crucial component of the glomerular filtration barrier (Pavenstadt, 2000). Podocytes are highly specialised cells that can be divided into three structurally and functionally different

segments: the cell body, the major processes, and foot processes interlinked by slit diaphragms.

In addition to podocytes the renal corpuscle contains mesangial cells, which are responsible for phagocytosis, in order to maintain a clean GBM, structural support of the podocytes, and secretion of a variety of molecules (Martini and Bartholomew, 1997). These cells are located between the individual capillaries of the glomerular tuft.

The rest of the nephron is composed of the renal tubule that from the Bowman's capsule can be divided into four sections: the proximal tubule (including convoluted and straight parts), the thin limbs of the loop of Henle, the distal tubule (including convoluted and straight portions), and finally the connecting segment that empties into the collecting duct (Vize *et al.*, 2003).

The different cell types of the kidney express specific proteins that can serve as markers for their identification.

Synaptopodin is an actin-associated protein that is characteristic of differentiated podocytes (Mundel *et al.*, 1997a). WT1, which is expressed in MM and in MM derivatives at different stages of maturation, is also upregulated in mature podocytes. It is expected that mature podocytes would therefore present a high expression level of WT1 (Dressler and Douglass, 1992). Podocalyxin is highly synthesized in differentiating podocytes and is restricted to apical surfaces of foot processes and to the luminal membrane domain of endothelial cells (Schnabel *et al.*, 1989; Vize *et al.*, 2003), but it is not specific for podocytes because it is also expressed by endothelial cells (Shankland *et al.*, 2007). Neph1 expression is

restricted to lateral margins of podocyte foot processes at the insertion of the slit diaphragm (Barletta *et al.*, 2003).

CD2-associated protein (CD2AP) is expressed in mature kidney in podocytes, however, it is also expressed in proximal tubule, and collecting duct and therefore cannot be considered podocyte specific (Akilesh *et al.*, 2007).

Vascular endothelial growth factor (VEGF) is involved in migration, proliferation, and differentiation of the vasculature of the kidney (Vize *et al.*, 2003). In kidney development VEGF is expressed in condensed mesenchyme and portions of the S-shaped body, and with kidney maturation becomes restricted to the glomerular epithelium and tubular cells (Simon *et al.*, 1995; Tufro *et al.*, 1999; Vize *et al.*, 2003). Developing podocytes also secrete VEGF, which guides endothelial cells towards developing glomeruli (Guan *et al.*, 2006) and even after glomeruli are fully mature podocytes continue to synthesize VEGF (Vize *et al.*, 2003).

Vimentin, an intermediate filament protein of the cytoskeleton, is expressed in early kidney development in the undifferentiated metanephric mesenchyme, however, as the meschymal cells begin to differentiate its expression is lost. Later, the developing podocytes begin to express vimentin again, and as they develop its expression becomes restricted finally to their bodies and major processes (Vize *et al.*, 2003). Podocytes can also be identified morphologically when cultured *in vitro.* Differentiated podocytes *in vitro* are large cells with a large cytoplasmic to nuclear volume ratio, arborized and often binucleated (Saleem *et al.*, 2002; Shankland *et al.*, 2007).

The mesgangial cells of the glomerulus can be identified by their expression of the markers α smooth muscle actin (αSMA) and desmin. αSMA is a marker of smooth muscle cells that it is expressed in mesangial cells during kidney development and is also present in high amounts in vascular smooth muscle cells (Skalli *et al.*, 1989; Stephenson *et al.*, 1998). Similarly, Desmin is also expressed in mesangial cells as well as in vascular smooth muscle cells (Zou *et al.*, 2006).

Some general epithelial markers, such as Kidney specific cadherin (Ksp-cad) and cytokeratins, can be used to identify cells of the tubular epithelium. KSP-cad is expressed in renal tubular epithelial cells, and is the only member of the cadherin family that it is exclusively found in the kidney. Cytokeratins are cytoskeletal proteins that form part of the intermediate filament group, and are characteristic of epithelial cells (Fleming and Symes, 1987). Cytokeratin 8 is expressed in all parts of the developing nephron and in adult tissue is expressed in all tubule epithelial cells (Oosterwijk *et al.*, 1990).

Other markers are specifc for certain portions of the nephron. Aquaporin 1 (AQP1) is expressed in kidney proximal tubules and descending thin limbs (Jenq *et al.*, 1999), and megalin (Oliver *et al.*, 2002) and alkaline phosphotase (AP) are known to be expressed in proximal renal epithelial cells (Baer *et al.*, 1999; Bussolati *et al.*, 2005; Sagrinati *et al.*, 2006). Meanwhile, Tamm-Horsfall protein (THP) is localized in the thick ascending limb of the loop of Henle and the early distal convoluted tubule (Chakraborty *et al.*, 2004). The collecting ducts can be identified by their expression of aquaporin 2 (AQP2) (Cai *et al.*, 2005).

Zonula Occludens 1 (ZO-1) is expressed in the tight junctions of epithelial cells and in the kidney can be found in the epithelial foot processes, in particular being concentrated at the points of insertion of the slit diaphragms into the lateral cell membrane (Schnabel *et al.*, 1990). While its expression is not specific to a particular portion of the tubular structure of the kidney its relative expression levels can be used to identify certain portions. ZO-1 is in epithelia throughout the tubular fractions of the kidney but stronger expression is observed in the distal segments (Gonzalez-Mariscal *et al.*, 2000).

Endothelial markers as platelet endothelial cell adhesion molecule (PECAM) (Redick and Bautch, 1999) and von Willebrand factor (vWF) (Pusztaszeri *et al.*, 2006) were also studied on the cells.

A number of groups have attempted to integrate embryonic stem cells or kidney stem cells into developing kidneys in order to evaluate whether the developing kidneys can provide an adequate environment and the signals required for the stem cells to differentiate into renal cells or even integrate into the developing structures (Kim and Dressler, 2005; Maeshima *et al.*, 2006; Steenhard *et al.*, 2005).

When injected into developing kidneys *ex vivo*, embryonic stem cells have successfully formed tubular epithelial structures (Kim and Dressler, 2005; Steenhard *et al.*, 2005). Maeshima *et al.* (2006) isolated renal progenitors from rat kidney and injected them into embryonic kidney *ex vivo* and found that 40% of the injected cells integrated into various parts of the developing kidneys.

Differentiation protocols for kidney stem cells commonly include animal serum (foetal bovine serum, foetal calf serum or horse serum) in the differentiation medium, and it appears that serum has some role in promoting differentiation (Bussolati *et al.*, 2005; Challen *et al.*, 2006; Kruse and Tseng, 1993; Sagrinati *et al.*, 2006).

6.1.1 Aims

The aim of this chapter was to clarify whether the expanded population of Pax-2 positive renal cells were kidney stem cells. The putative stem cells were tested for their ability to differentiate into specific kidney cell types, and the resulting cells were investigated for cell marker expression in order to characterise them. The differentiated cells were also tested for their clonogencity. Finally, the cells were tested for their ability to integrate into a developing kidney, in order to evaluate their nephrogenic potential.

6.2 Results

6.2.1 In vitro differentiation of KSC

In order to promote differentiation of KSC into renal-specific cell types, cells grown on fibronectin for eleven passages were plated into uncoated plastic dishes and STO-CM was changed to DMEM containing serum (10% FCS). The cells were grown for five passages (one month) under these conditions. The cells were then analysed for MM and renal-specific markers by immunostaining and RT-PCR, and changes in their morphology were evaluated. Hereafter cells that had been cultured under the differentiating conditions will be referred to as

'differentiated putative kidney stem cells' (dpKSC) to distinguish them from those maintained under the original conditions (pKSC).

Morphological analysis

When in culture the pKSC appeared as a relatively homogeneous population, however, the dpKSC population became more heterogeneous and cells with different morphologies were observed (Figure 6-1). Some cells formed layers of epithelial-like cells, some became spindle shaped or mesangial-like cells with their characteristic stellate morphology (Cortes-Hernandez *et al.*, 2002). Others displayed a cobblestone morphology characteristic of immature podocytes in culture (Shankland *et al.*, 2007). Arborized, binucleated cells, which are characteristic of differentiated podocytes, were also observed. However, in the colonies some cells with morphology similar to undifferentiated pKSC were observed.

Investigation of MM-specific marker expression

It has been demonstrated that the pKSC maintained expression of the metanephric mesenchymal markers Pax-2 and WT1 when cultured through a number of passages, indicating that they may have been stem cells. In order to determine whether the cells differentiated under the new culture conditions, the expression of these markers was tested in dpKSC. After five passages in the differentiating conditions, the cells were immunostained for Pax-2 and WT1. While pKSC maintained expression of Pax-2 and WT1, dpKSC continued to express WT1, but the majority of the cells lost Pax-2 expression (Figure 6-2).

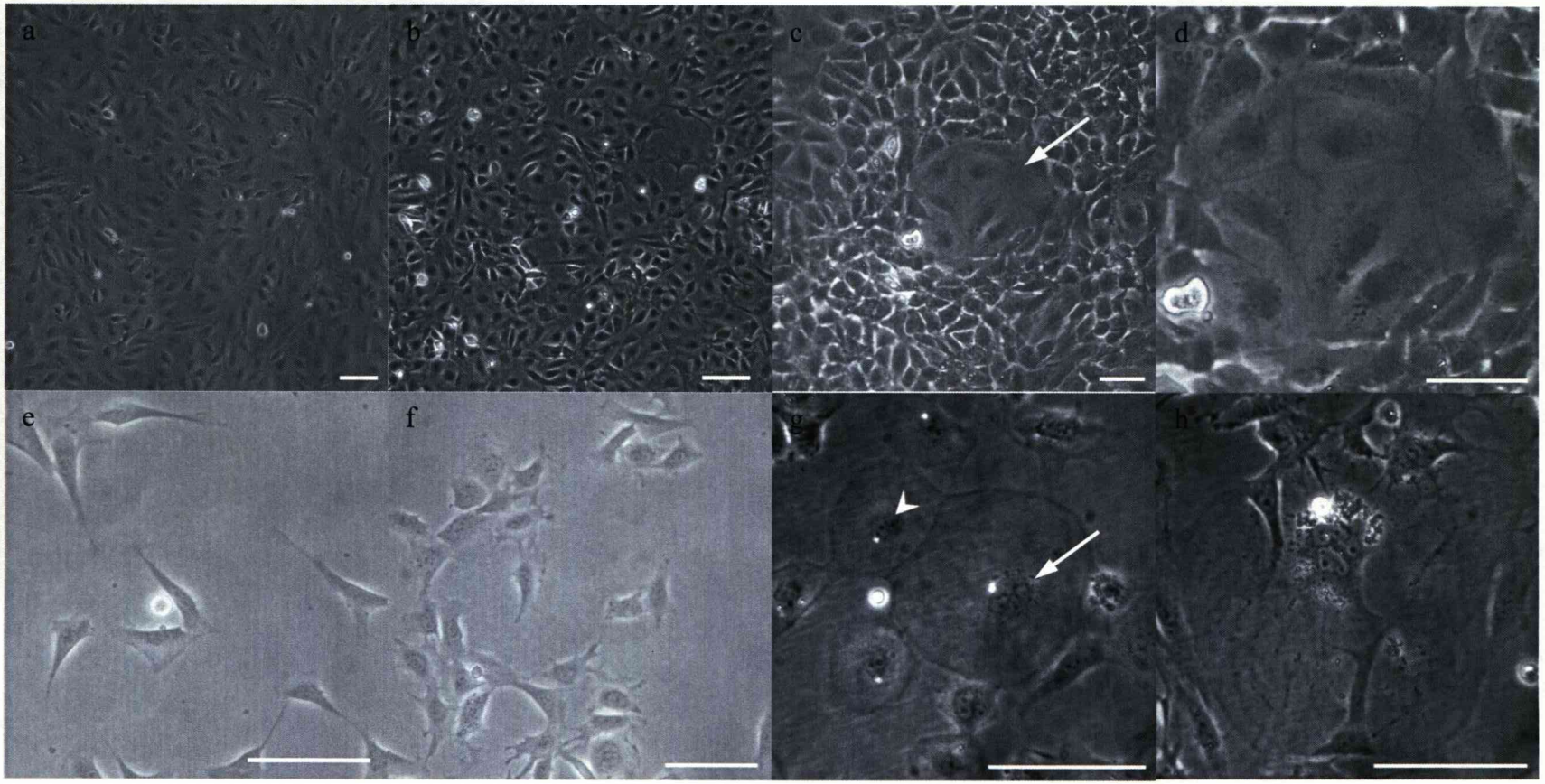


Figure 6-1 Phase contrast pictures showing different morphologies of differentiated pKSC and dpKSC. (a) Homogenic morphology of pKSC. (b) Heterogenic morphology of dpKSC. (c) A group of non differentiating cells and a group of epithelial-like cells (arrow) showing tight junctions between them. (d) Higher magnification image of the epithelia-like cells showed in (c). (e) Cells with spindle morphology. (f) Cells showing a mesangial-like morphology. (g) Podocyte-like binucleated cell (arrows) and other cell, possibly immature podocytes (arrowshead). (h) Podocyte-like binucleated cells with large cytoplasmic volume and interdigitating processes in cytoplasm. Scale bars:

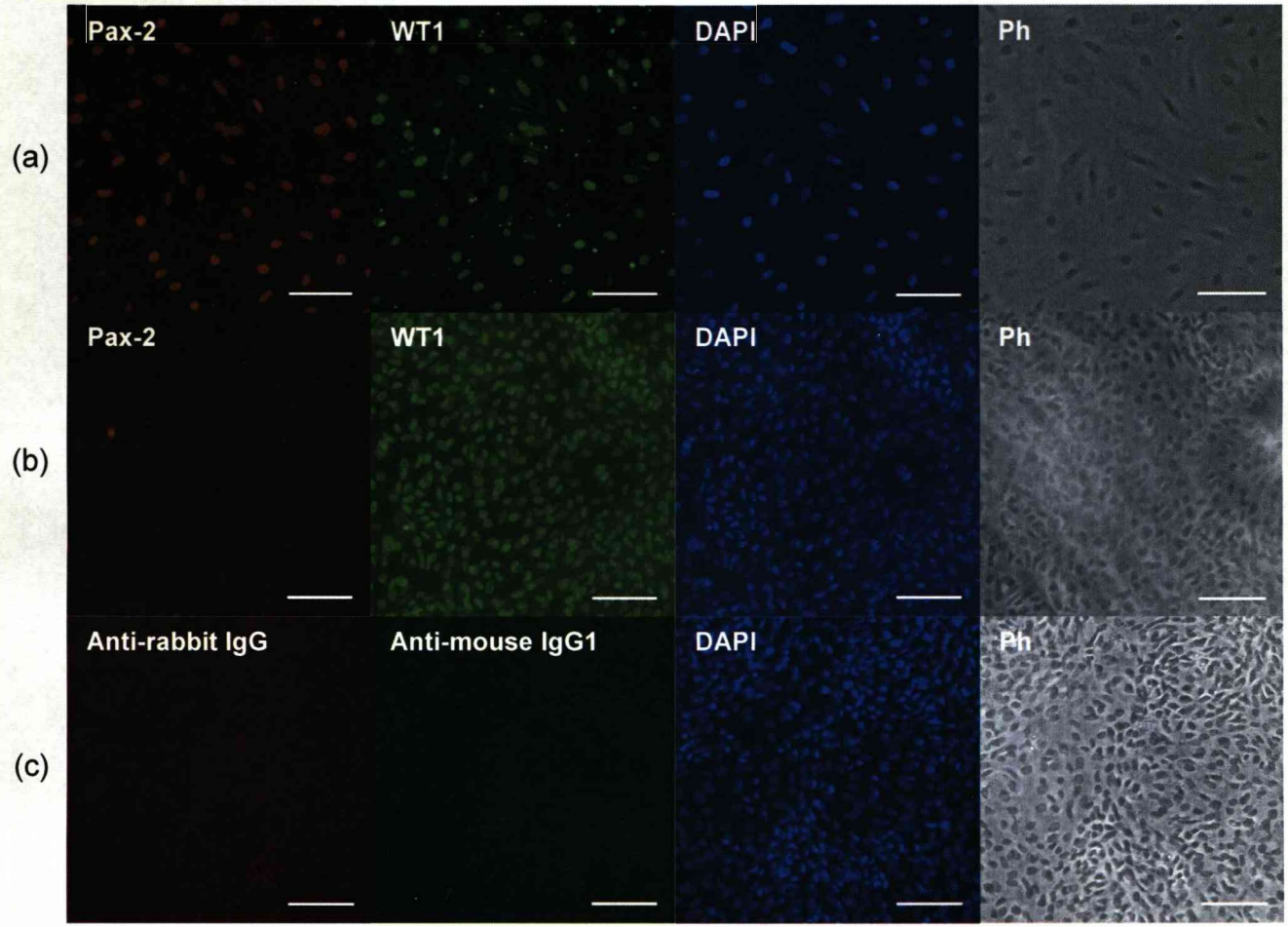


Figure 6-2 Micrographs showing immunostaining for Pax-2 and WT1 of pKSC and dpKSC. (a) pKSC maintained Pax-2 and WT1 expression in culture. (b) dpKSC maintained WT1 expression but lost expression of Pax-2. (c) Negative control dpKSC with primary antibody omitted from stain. Scale bars 100 μm. Ph – phase contrast.

RT-PCR was performed to detect expression of Pax-2, WT1 GDNF and Sall1. The results showed that WT1 and GDNF expression was maintained following differentiation but, as expected from the immunostaining results, there was no Pax-2 expression (Figure 6-3). This experiment was repeated and the results of both immunostaining and RT-PCR were shown to be reproducible.

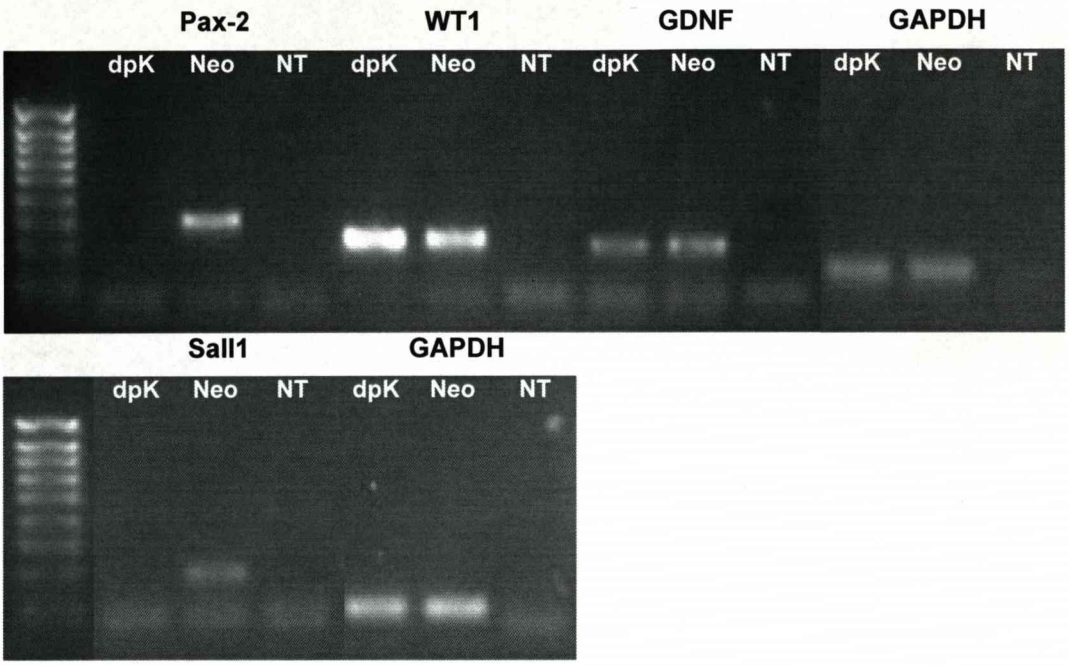


Figure 6-3 RT-PCR analysis of dpKSC for Pax-2, WT1, Sall1, GDNF and GAPDH. dpKSC (dpK) did not express Pax-2 or Sall1, but were positive for WT1 and GDNF. Positive control in each case was 5 day old kidney (Neo); no template control (NT) also shown.

Investigation of renal-specific marker expression

To determine whether the cells were differentiating to renal-specific cell types a variety of renal-specific markers were investigated using RT-PCR and immunostaining.

Expression of the specific renal marker synaptopodin was investigated to determine whether the dpKSC were assuming podocyte commitment. Furthermore, WT1 expression levels were evaluated as it is known that WT1 is

more highly expressed in mature podocytes than in MM cells (Ryan *et al.*, 1995). When dpKSC were stained for synaptopodin and WT1 clusters of cells could be observed that showed higher levels of WT1 expression and higher levels of synaptopodin expression (Figure 6-4a). These cells also presented cytoplasmic synaptopodin.

Among the dpKSC some binucleated cells were present. These binucleated cells also had a higher cytoplasmic volume compared with the rest of the cells in culture. WT1 expression in these cells was higher than in neighbouring cells; this would be consistent with them being podocytes (Figure 6-4c). Both pKSC and dpKSC were positive for synaptopodin when they were analysed by RT-PCR (Figure 6-5). Other podocyte markers such as podocalyxin, neph1 and CD2AP were positive in pKSC and dpKSC when they were analysed by RT-PCR (Figure 6-5).

In order to check whether mesangial cells were founded among the differentiated cells, the mesangial marker αSMA (alpha smooth muscle actin) was used to stain dpKCS. When pKSC were stained for αSMA most of the cells appeared negative for αSMA with no staining observable above background levels (Figure 6-6a). However, many dpKSC were found to be αSMA positive (Figure 6-6b and c). No positive staining was found in the dishes where the primary antibody had been omitted (Figure 6-6d).

The mesangial marker Desmin was expressed in pKSC and dpKSC in RT-PCR. Another cytoskeletal protein studied by RT-PCR was vimentin which was expressed in pKSC and in dpKSC (Figure 6-5).

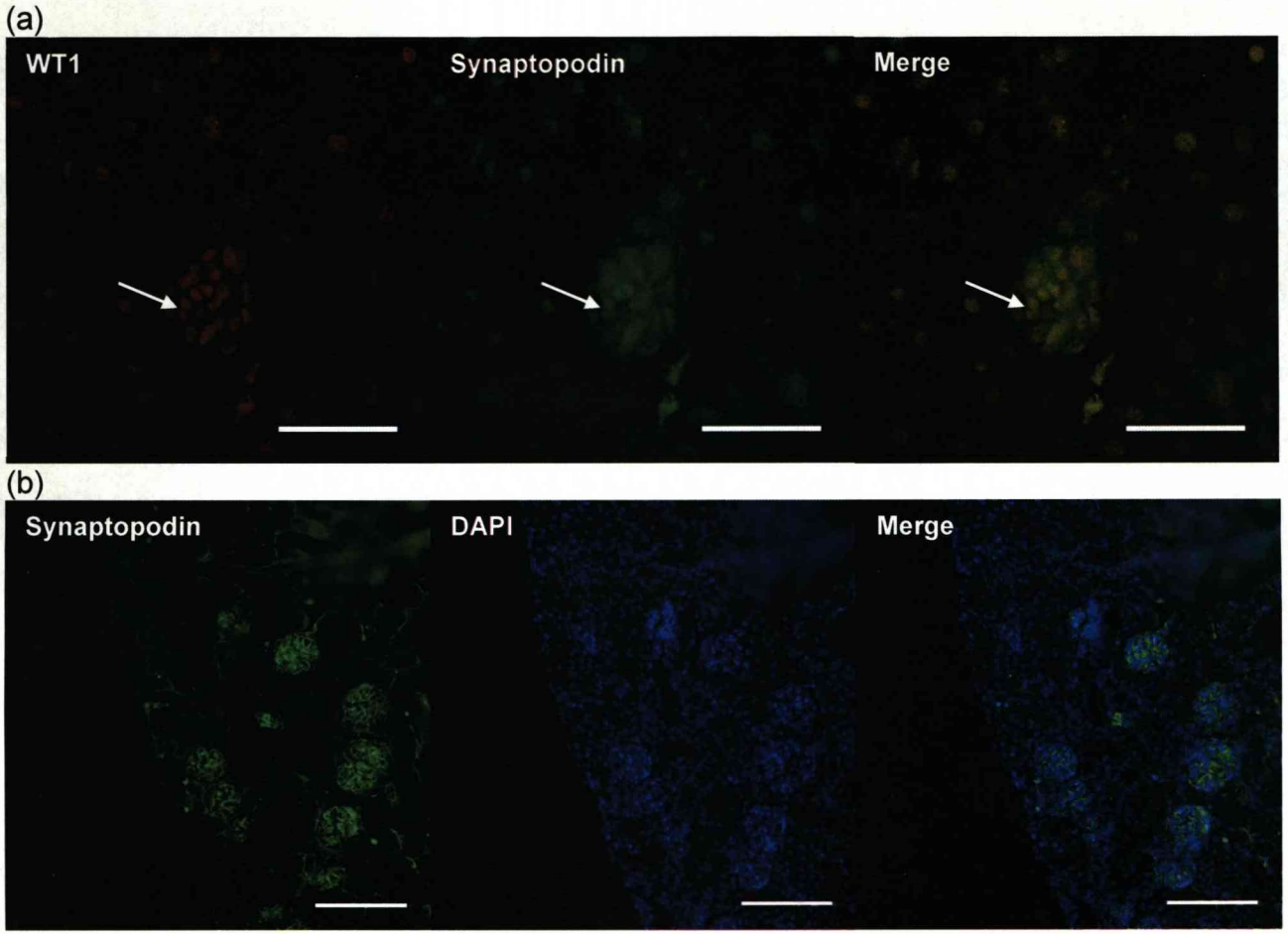


Figure 6-4(a & b) Immunostaining of dpKSC for WT1 and Synaptopodin. (a) Clusters of cells with high WT1 expression also had high Synaptopodin expression (arrow). (b) Neonate kidney showing Synaptopodin expression in glomeruli as positive control. Ph – Phase contrast. (c) Negative control in which primary antibody was omitted. Scale bars: 100 μm.

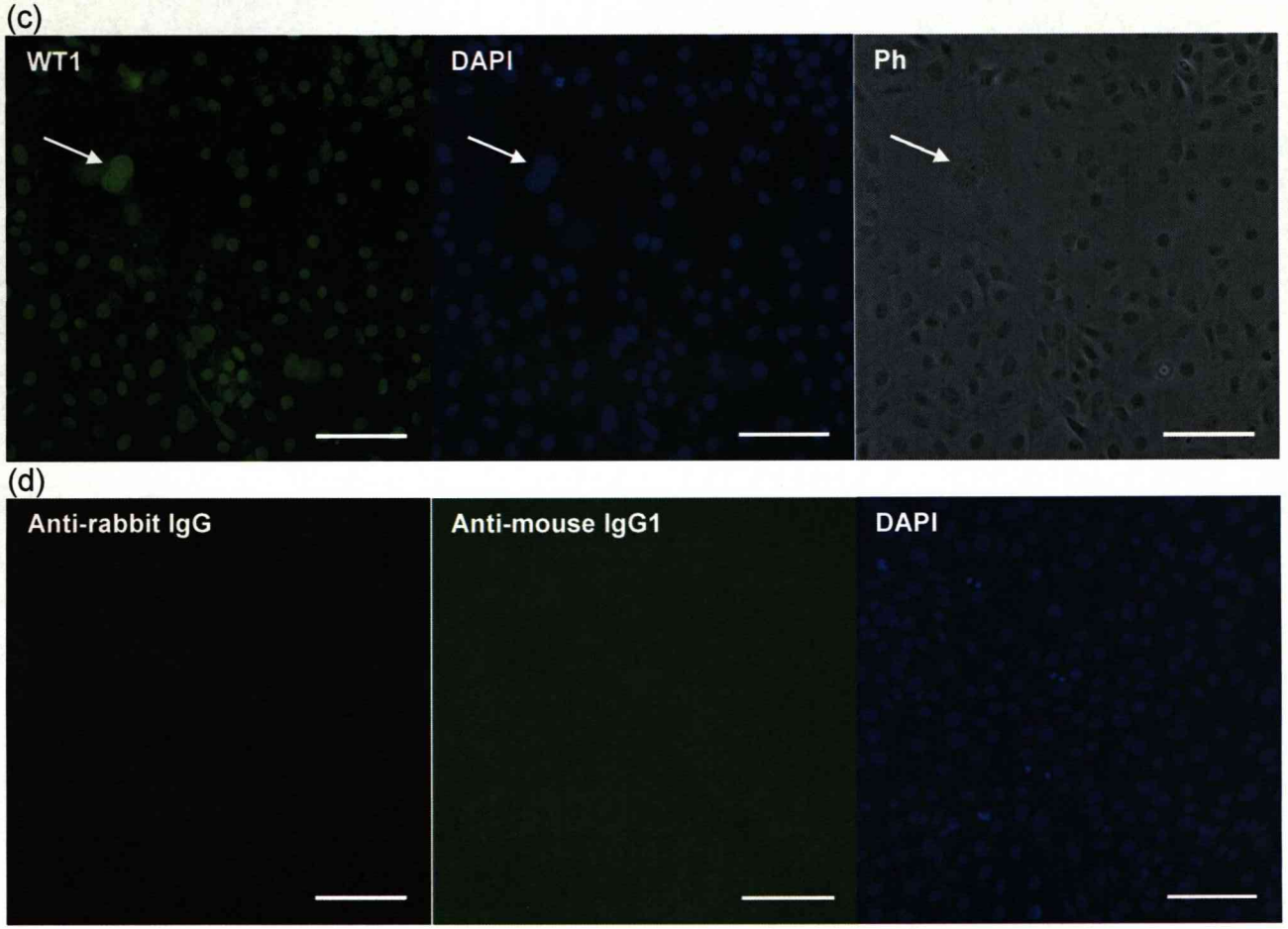


Figure 6-4(c & d) Immunostaining of dpKSC for WT1 and Synaptopodin. (c)Binucleated cells showed high WT1 expression (arrow). (d) Negative control in which primary antibody was omitted. Ph – Phase contrast. Scale bars: 100 µm.

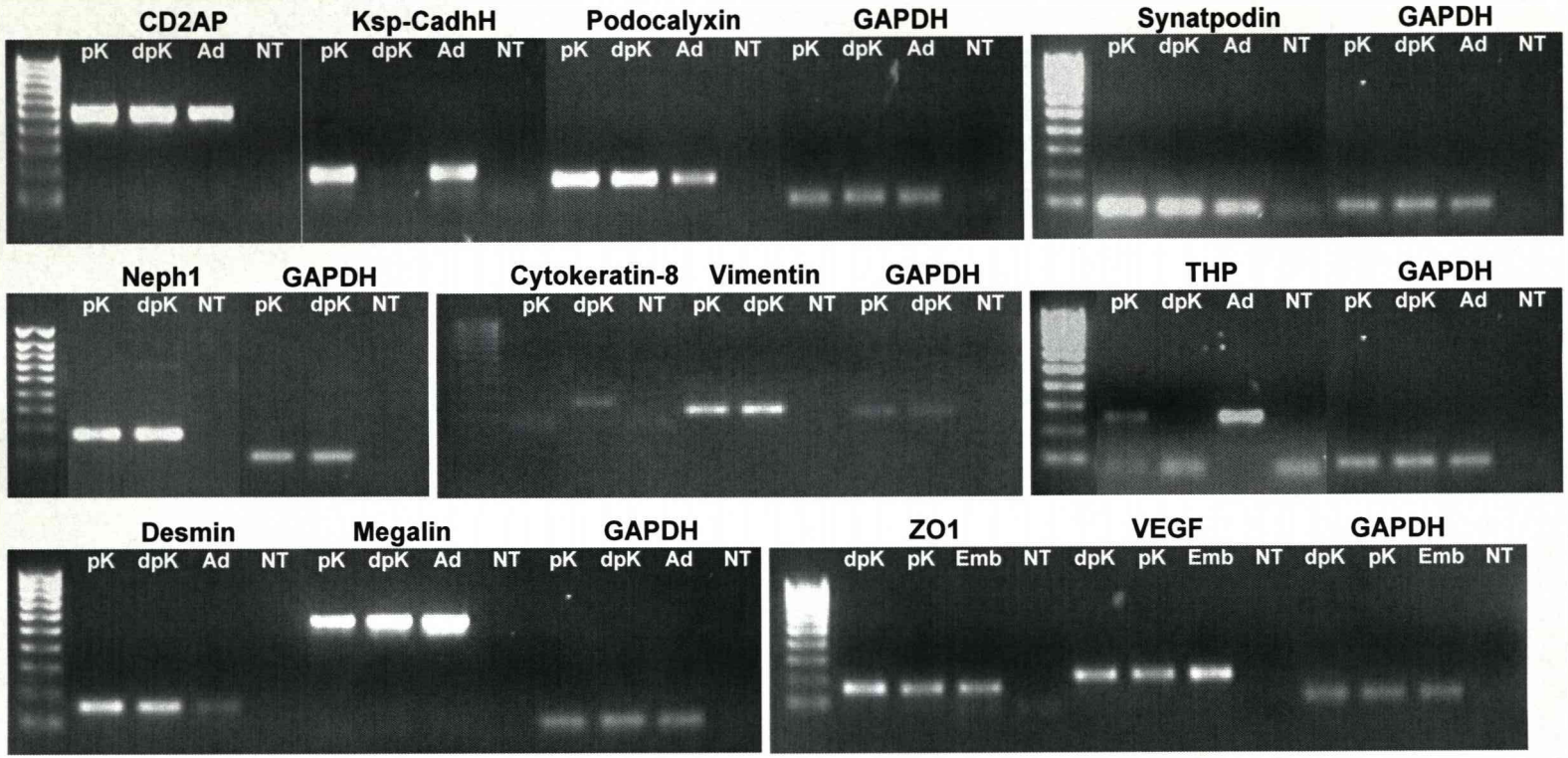


Figure 6-5 RT-PCR of pKSC and dpKSC for kidney markers. The results of the RT-PCRs shown for pKSC (pK) and dpKSC (dpK) are summarised in Table 6-1. Kidneys from E13 embryo (Emb) and adult (Ad) were used as positive controls, and reactions run with no template (NT) as negative controls.

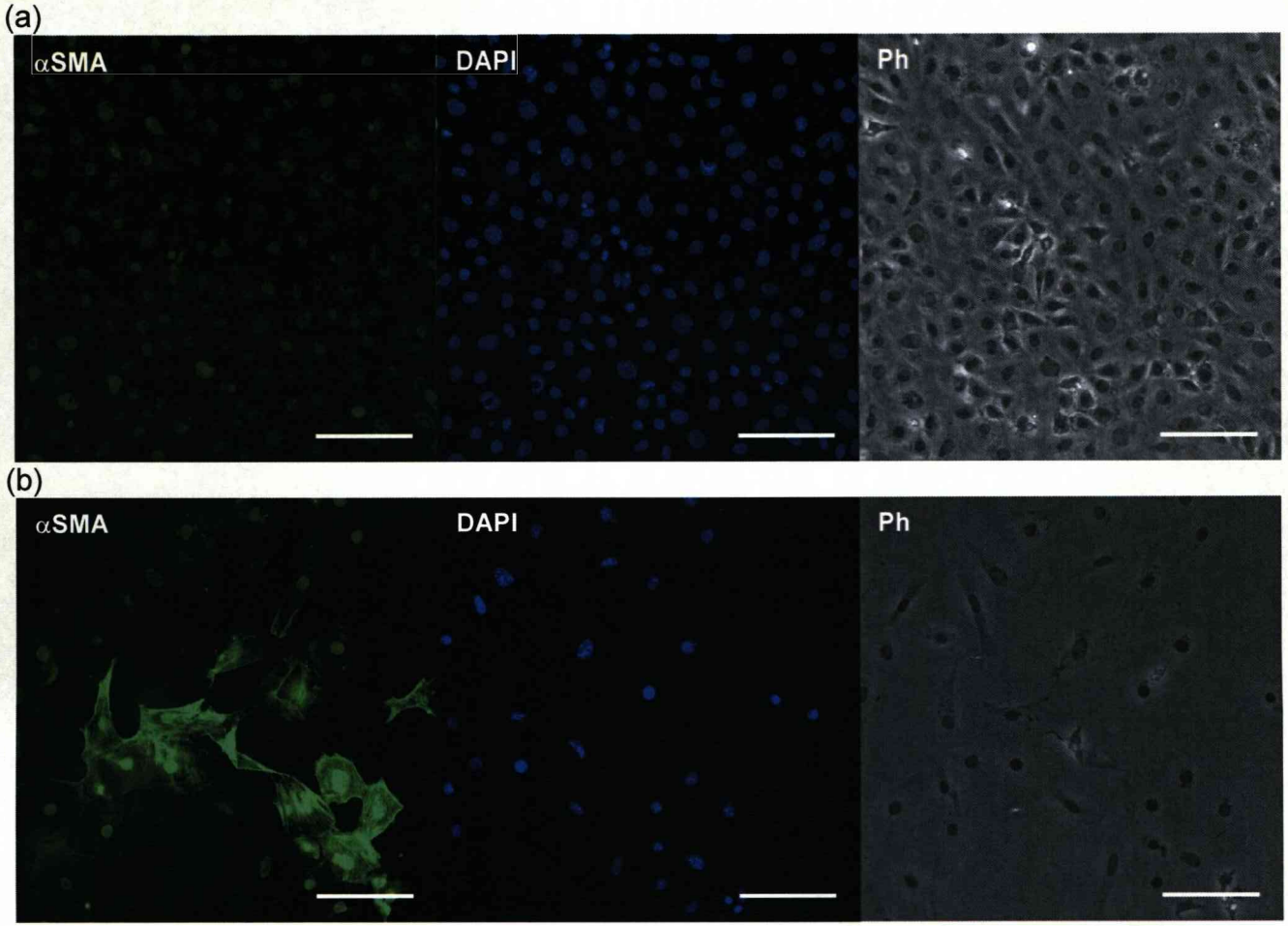


Figure 6-6(a & b) Immunostaining of pKSC and dpKSC for the mesangial marker αSMA. (a) pKSC showed no expression of αSMA. (b) Some dpKSC expressed αSMA. Scale bars: 100 μm.

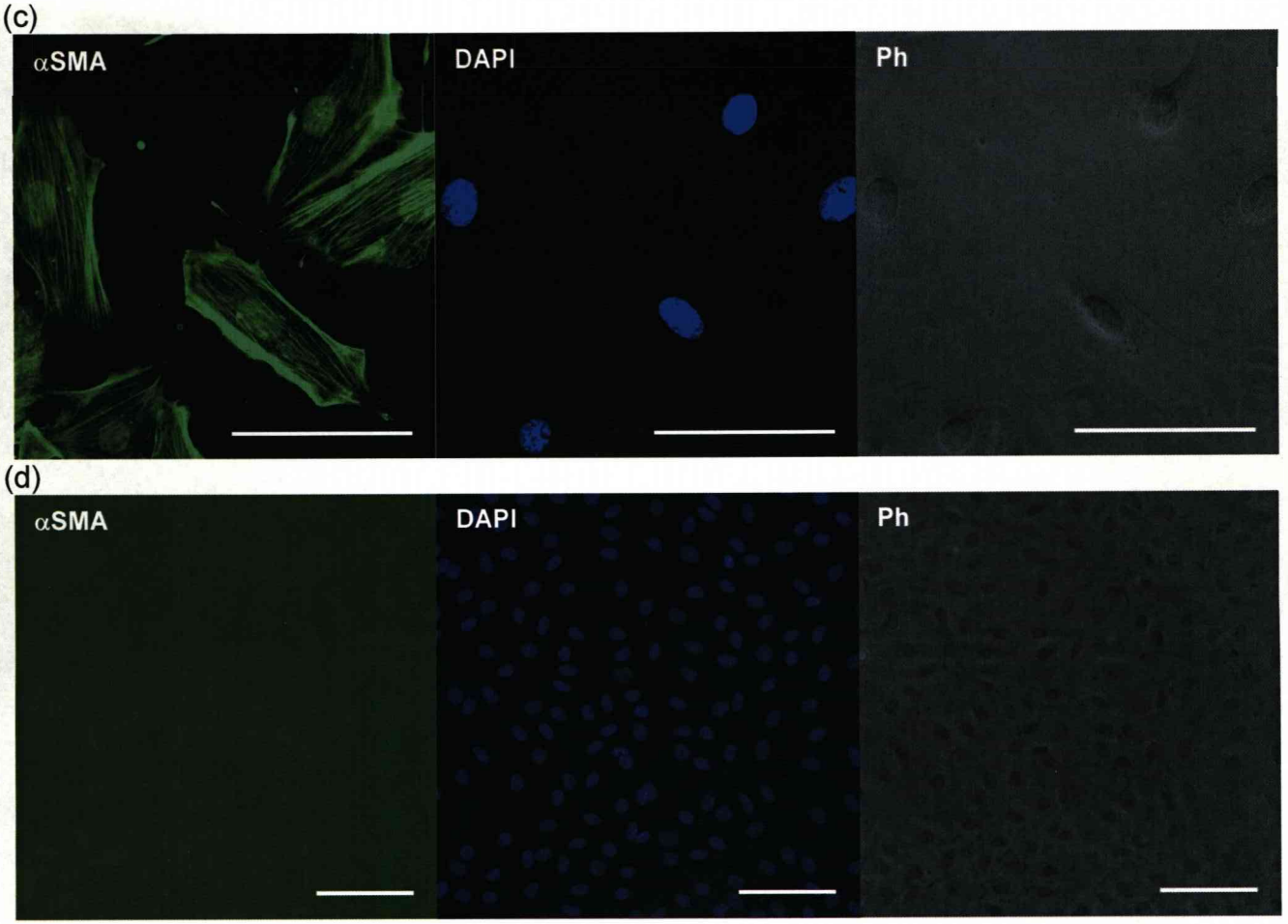


Figure 6-6(c & d) **Immunostaining of pKSC and dpKSC for the mesangial marker αSMA. (c) Higher magnification shows association of αSMA with the cytoskeleton in dpKSC. (d) Negative control in which primary antibody was omitted from stain. Scale bars: 100 μm.**

In order to determine whether there were epithelial cells among the dpKSC, the cells were stained for cytokeratin expression. Very few pKSC cells presented cytokeratin expression, however, clusters of the dpKSC were positive for cytokeratin (Figure 6-7). No specific staining was observed in samples where primary antibody was omitted.

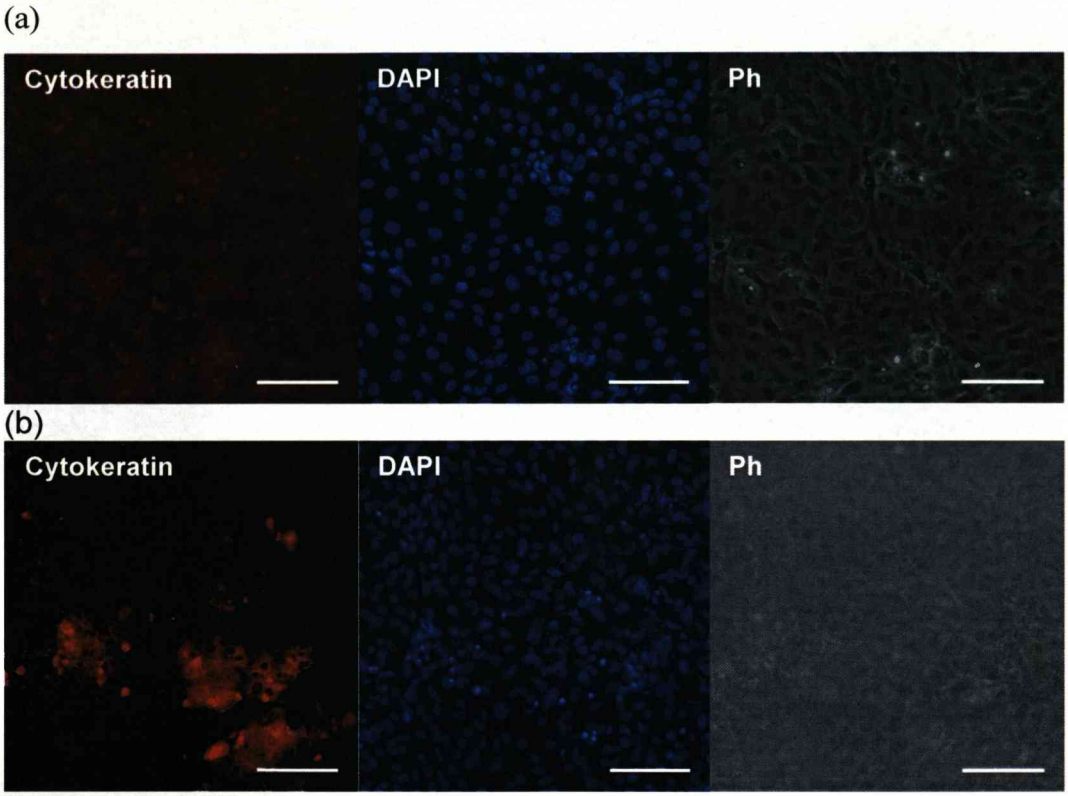


Figure 6-7 Immunostaining of pKSC and dpKSC for the epithelial marker cytokeratin. (a) pKSC were not shown to express cytokeratin. (b) Cytokeratin staining of dpKSC showed some clusters positive for the marker. Scale bars: 100μm. Ph – phase contrast.

The epithelial marker cytokeratin-8 was not expressed in pKSC, but was expressed in dpKSC. The kidney specific cadherin, ksp-cadherin, was expressed in pKSC but not in dpKSC (Figure 6-5).

Based on the morphology it was hypothesised that the epithelial-like cells present in the differentiated cultures could be proximal or distal tubular cells of the

nephron. To determine whether proximal tubular cells were present, cultures were stained for the presence alkaline phosphatase. While no alkaline phosphatase activity was detected in the pKSC, the dpKSC showed clusters of cells showing AP activity (Figure 6-8). The cells showing AP staining presented an epithelial-like morphology. Some of the cells with no AP activity showed morphology characteristic of podocytes.

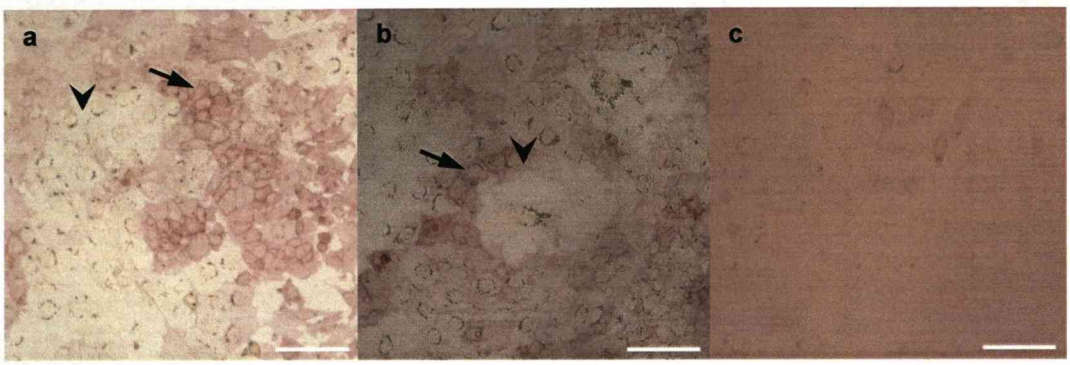


Figure 6-8 Alkaline phosphatase staining of dpKSC. (a) dpKSC formed layers of epithelial-like cells that were positive for alkaline phosphatase (arrow), while cells negative for AP did not exhibit epithelial-like morphologies (arrowhead). (b) Some dpKSC that were negative for AP exhibited binucleated podocyte-like morphologies (arrowhead); arrow indicates AP positive epithelial-like cells. (c) pKSC were negative for AP expression. Scale bars: 100 μm.

To confirm that proximal tubular cells were present, the cells were immunostained for the water channel Aquaporin 1 (AQP1). pKSC and dpKSC were negative for AQP1 staining (Figure 6-9). Staining for AQP1 was found in the one day old neonatal kidney included as a positive control. No staining was observed in the cells where the primary antibodies were omitted.

pKSC and dpKSC were positive by RT-PCR for the proximal tubular marker megalin (Figure 6-5).

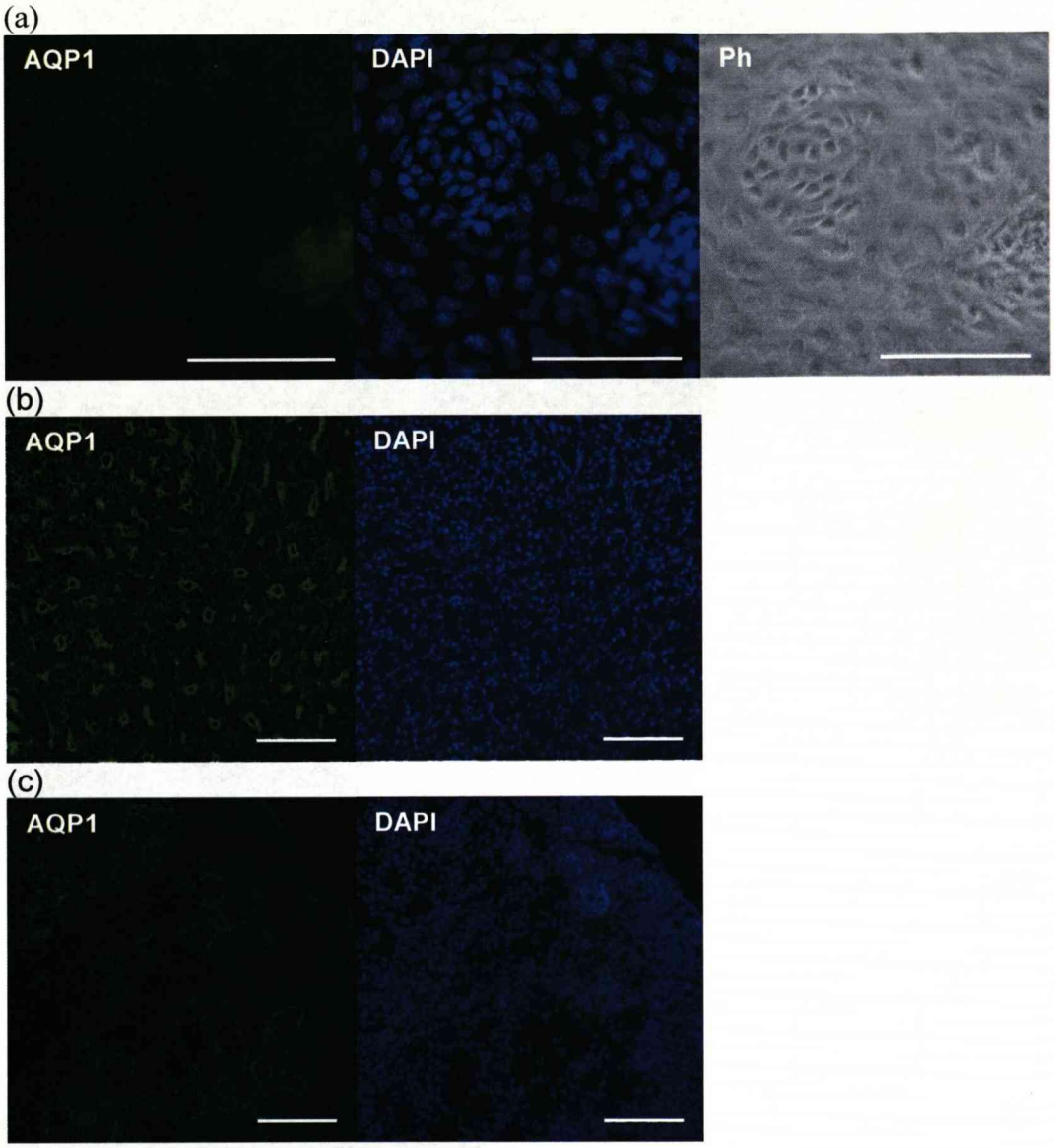


Figure 6-9 Immunostaining of dpKSC for aquaporin 1 (AQP1). (a) dpKSC did not express AQP1. Ph – Phase contrast. (b) Positive control – one day old kidney. (c) Negative control in which primary anitbody was omitted from stain – one day old kidney. Scale bars: 100 μm

Other tubular markers were analysed by RT-PCR such as ZO-1 and THP, which are mostly expressed in distal portion of the nephrons (Figure 6-5). pKSC and dpKSC expressed ZO-1.

RT-PCR also showed that pKSC expressed THP, while dpKSC did not.

The cells were also immunostained for the water channel AQP2 expressed in collecting ducts. pKSC and dpKSC were negative for AQP2 staining (Figure 6-10). Staining for AQP2 was found in one day old neonatal kidney, which was included as a positive control. No staining was observed in the cells where the primary antibodies were omitted.

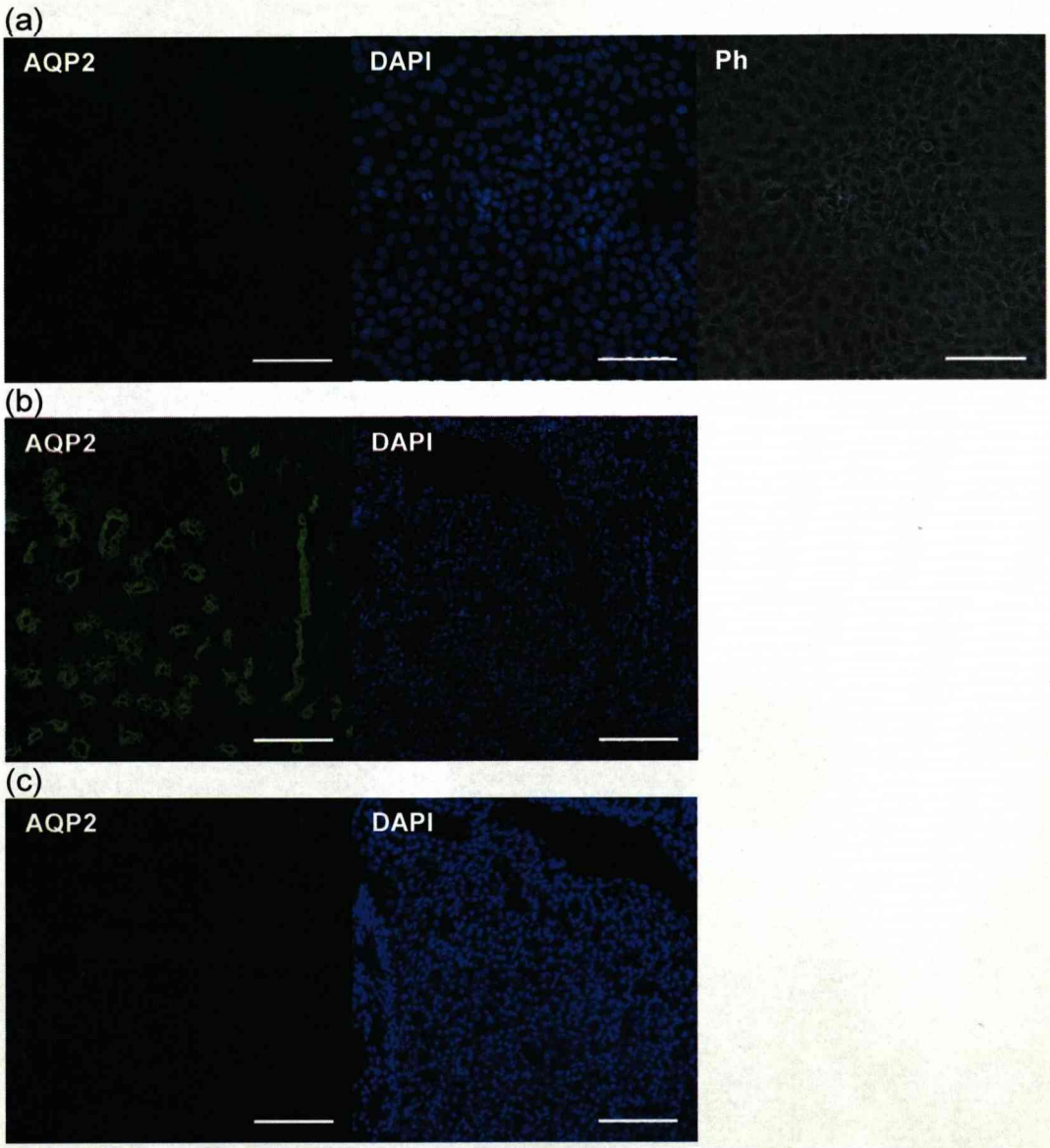


Figure 6-10 Immunostaining of dpKSC for aquaporin 2 (AQP2). (a) dpKSC did not express AQP2. Ph – Phase contrast. (b) Positive control – one day old kidney. (c) Negative control in which primary anitbody was omitted from stain – one day old kidney. Scale bars: 100 μm.

The dpKSC were also tested for the expression of two endothelial markers vWF and PECAM. No PECAM staining was found in pKSC. dpKSC showed no positive staining for either vWF or PECAM (Figure 6-11 and Figure 6-12).No staining was found in the samples in which the primary antibody had been omitted.

The last of the endothelial marker analysed by RT-PCR was VEGF, which was expressed in pKSC and in dpKSC (Figure 6-5). Table 6-1 summarise the RT-PCR results for all sixteen markers tested.

Reverting the Phenotype of differentiated pKSC

In order to test whether the dpKSC could regain Pax-2 expression, the cells were replated under the original culture conditions of fibronectin and STO-conditioned medium and analysed after 6 passages in culture. RT-PCR and immunostaining showed that the cells did not recover Pax-2 expression when the conditions were reverted (Figure 6-13 and Figure 6-14).

6.2.2 Extrarenal differentiation

In order to determine whether pKSC or dpKSC had the potential to differentiate to any cell type other than renal, adipogenic differentiation was induced. After a week fat droplets, characteristic of adipocytes, were identified by eye in the cytoplasm of many of cells in all the dishes. In the controls a few isolated vacuoles were also identified (Figure 6-15).

After two weeks and three weeks the presence of the fat droplets in the majority of the cells was confirmed using oil red O staining (Figure 6-15). There was no

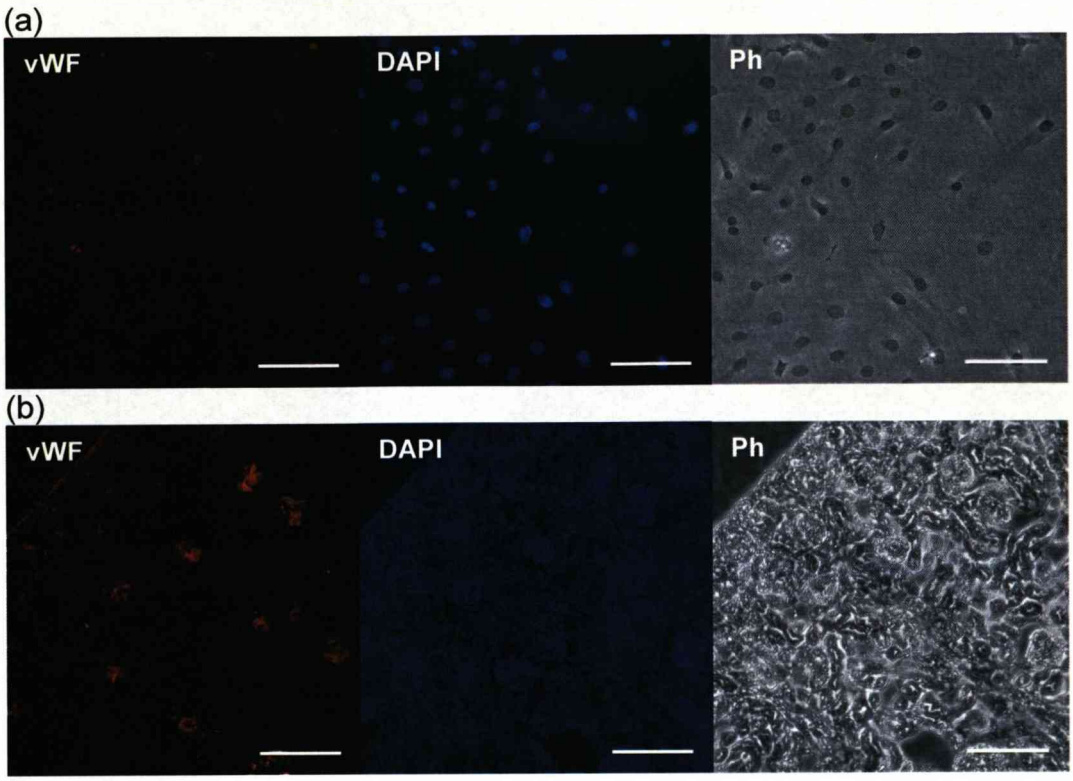


Figure 6-11 Immunostaining of dpKSC for von Willebrand Factor (vWF). (a) Immunostianing showed the absence of vWF in dpKSC. (b) Expression of vWF in neonatal mouse kidney was used as a positive control. Scale bars: 100 μm. Ph – Phase contrast.

Table 6-1 Summary of the RT-PCR results for pKSC and dpKSC.

Marker	pKSC	dpKSC
Pax-2	✓	×
WT1	✓	✓
GDNF	✓	✓
Sall1	✓	×
CD2AP	✓	✓
Ksp-Cadherin	✓	×
Neph1	✓	✓
Podocalixyn	✓	✓
Synaptopodin	✓	✓
THP	✓	×
Megalin	✓	✓
Desmin	✓	✓
Cytokeratin	×	✓
Vimentin	✓	✓
ZO-1	✓	✓
VEGF	✓	✓

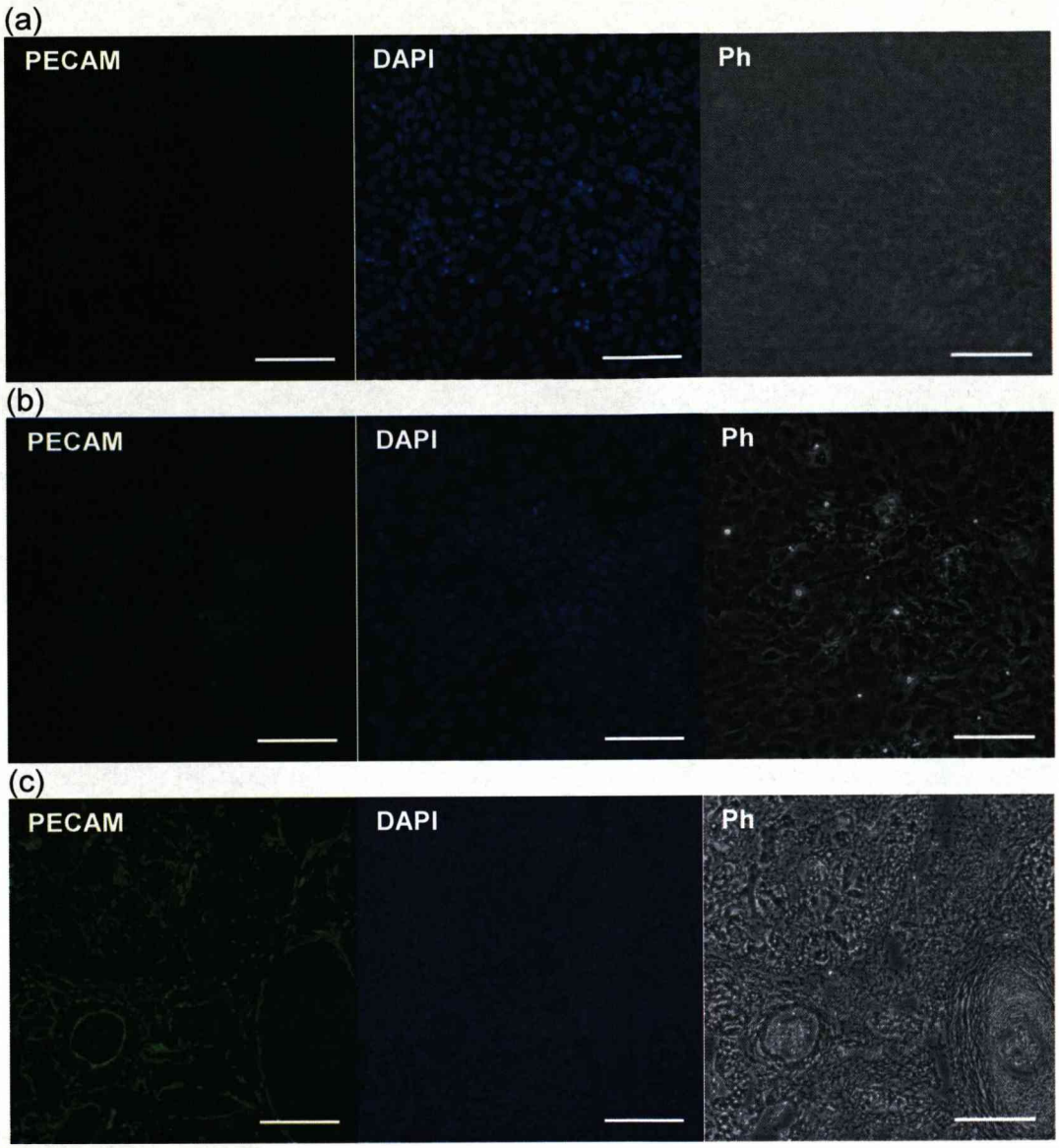


Figure 6-12 Immunostaining of pKSC and dpKSC for PECAM. (a) pKSC did not show any expression of PECAM. (b) dpKSC did not express PECAM. (c) Expression of PECAM in E14.5 mouse embryo as positive control. Scale bars: 200 μm. Ph – phase contrast.

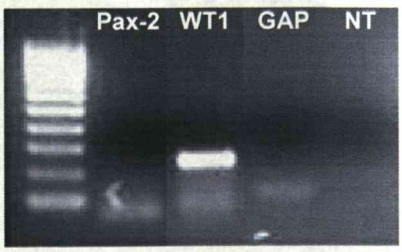


Figure 6-13 RT-PCR of dpKSC cells cultured for one month on fibronectin and STO-CM for Pax-2 and WT1. Moving the dpKSC from the differentiating conditions back to the renewal conditions did not result in the cells regaining expression of Pax-2; WT1 expression was maintained throughout.

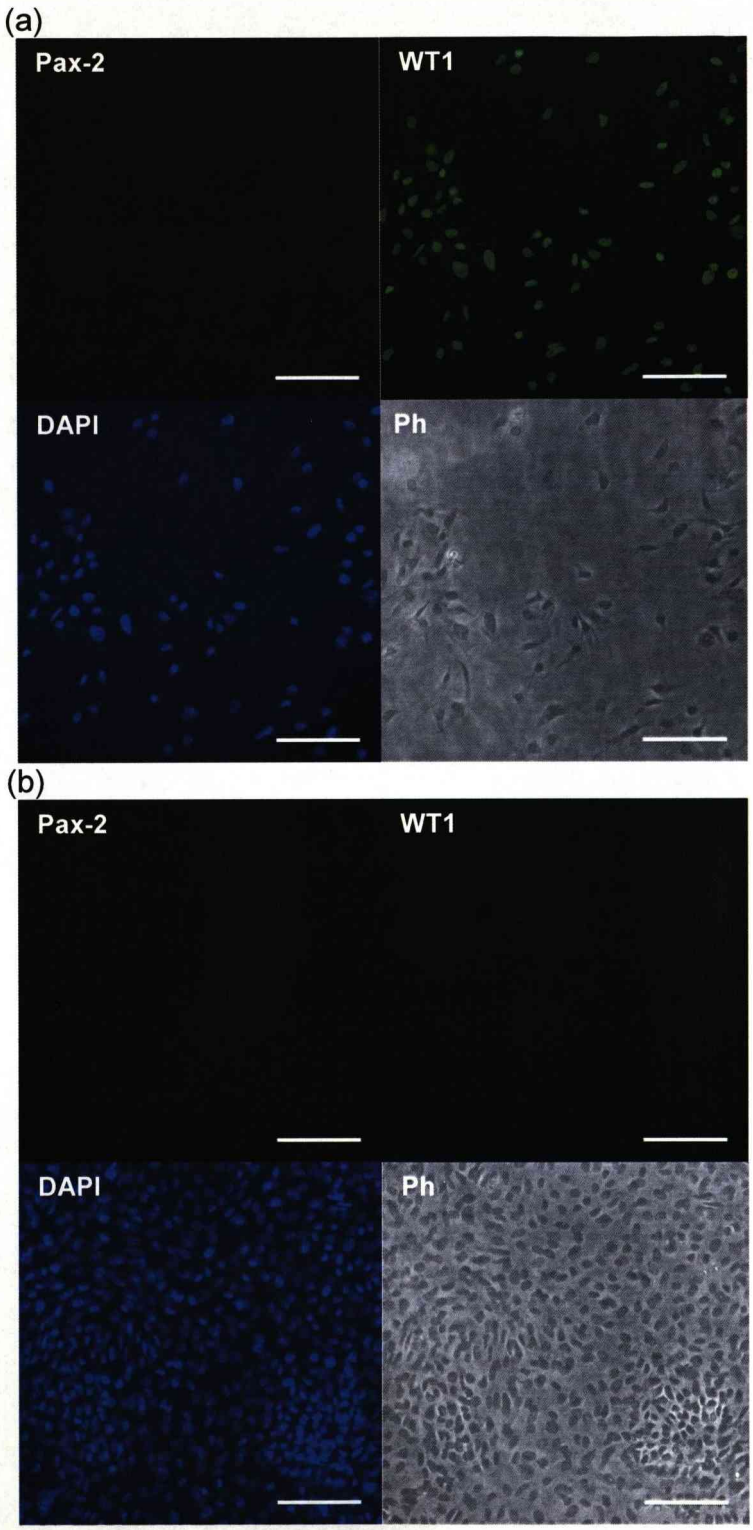


Figure 6-14 Immunostaining of dpKSC after reversion experiment for Pax-2 and WT1. (a) dpKSC cultured for 1 month in original 'renewal' conditions of fibronectin and STO-CM did not regain Pax-2, but did retain WT1 expression. (b) Negative control in which primary antibody was omitted from the stain. Scale bars: 100 μm. Ph- phase contrast.

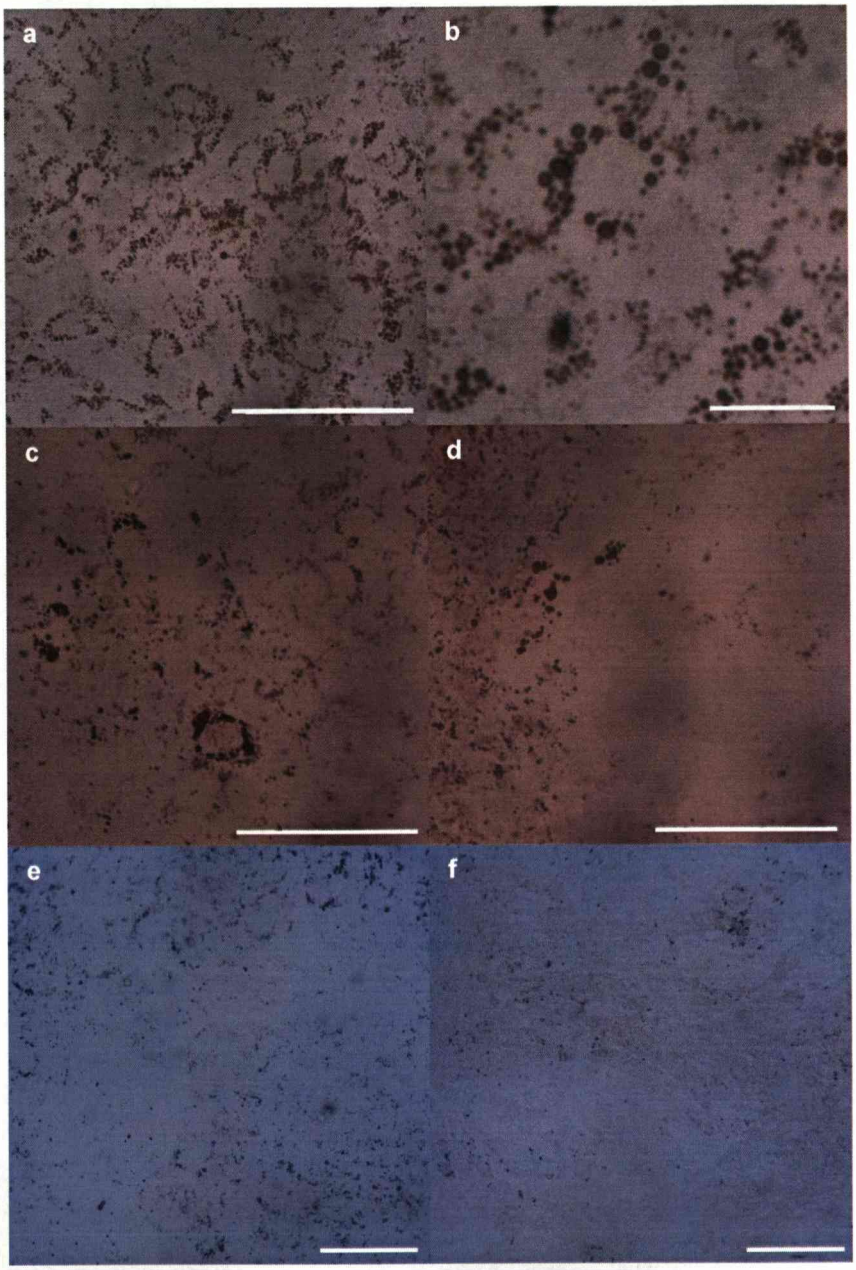


Figure 6-15 Adipogenic differentiation of pKSC. pKSC cultured for two and three weeks with adipogenic medium and stained with oil red O. (a) pKSC cultured with adipogenic medium for two weeks presented lipid droplets. (b) Detail of image (a) showing lipid droplets. (c) pKSC cultured for three weeks with adipogenic medium presented similar numbers of adipocytes. (d) dpKSC cultured for three weeks with adipogenic mediumshowed scattered cells with lipid droplets. (e) pKSC in control medium did not develop significant numbers of lipid droplets. (f) dpKSC in control medium did not develop significant numbers of lipid droplets. Scale bars 100 μm, except (b) where scale bar is 25 μm

increase in the number of cells with droplets from two to three weeks in the adipogenic conditions. Nearly all pKSC presented fat droplets, while in dpKSC cultures there were larger areas without fat droplets.

6.2.3 *Ex vivo* differentiation of KSC

In order to evaluate the nephrogenic potential of the expanded putative KSC, the potential of the pKSC to integrate into the developing kidneys of E11.5 mice was assessed. Calbindin was used to visualize ureteric bud structures. When the developing kidneys were stained with differentiation markers, such as synaptopodin or podocin, no staining was observed (data not shown). Most of the labelled KSC were found close to ureteric bud structures, and some were found condensed around the tips of the ureteric bud (Figure 6-16).

Clonogenic potential of KSC

When single cells were plated into Terasaki plates by mouth pipetting in CM-STO 30% attached, however, none proliferated and within 10 days all attached cells had died. By contrast when single cells were plated in 96 well plates by limiting dilution but in DMEM 10% FCS, many proliferated and formed colonies. From the total number of cells plated on the 96 well plates, 22 single cells were identified, and of these 17 formed colonies.

The clones from single cells were expanded (Figure 6-17). Differences in cellular morphology were observed between clones, but all the cells in an individual clone exhibited similar morphology (Figure 6-18a and b). Most of the clones presented

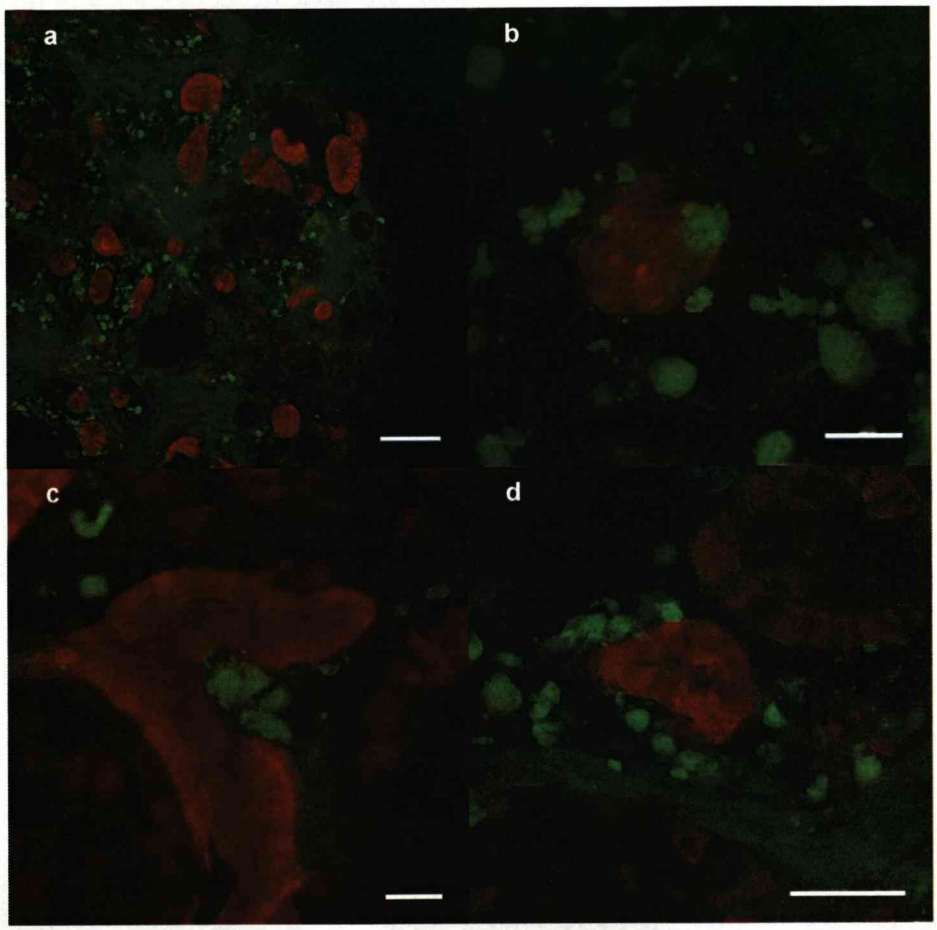


Figure 6-16 Immunostaining for calbindin in integration experiment of KSC-vybrant dye into developing kidneys. (a) Immunostaining for calbindin (red) showing general view of the whole developing rudiment where pKSC (green) are condensed around ureteric bud structures. (b-c) Immunostaining for calbindin showing pKSC condensed around ureteric bud tips. Scale bars: (a) 200 μm, (b) and (c) 20 μm and (h) 50 μm.

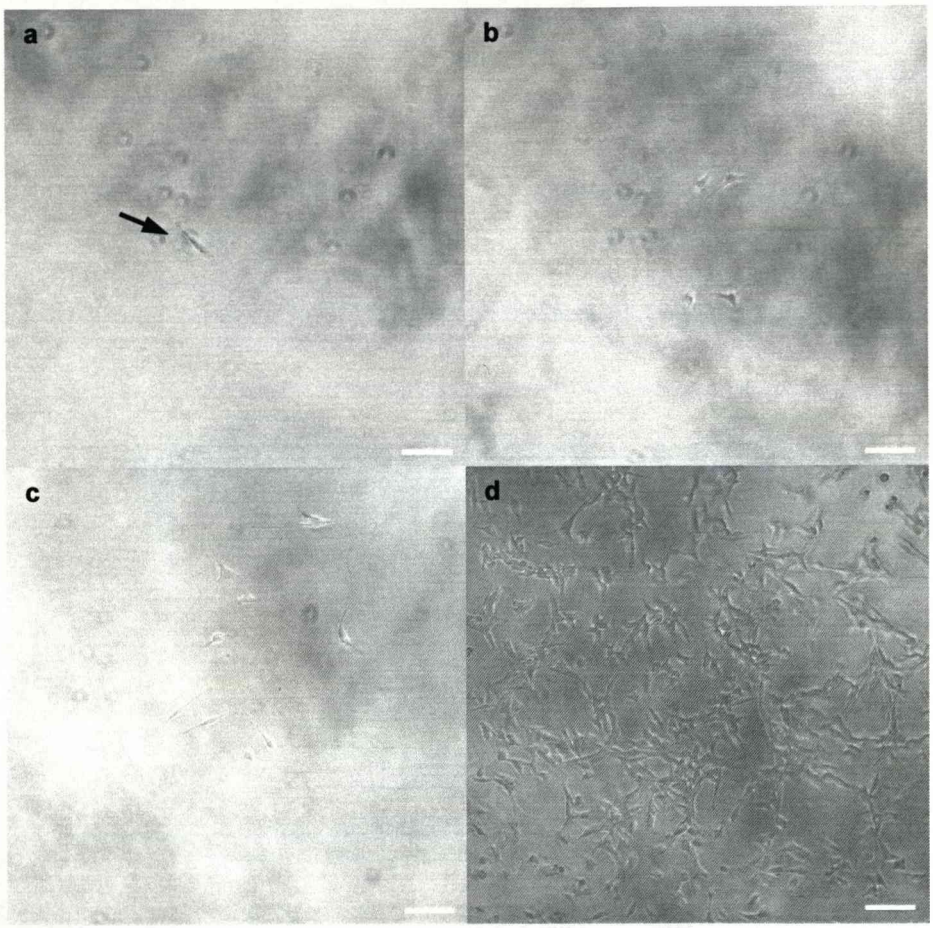


Figure 6-17 Bright field images showing time course expansion of one clone (C4). (a) Bright field images showing a single cell one day after plating. (b) Bright field image showing cells proliferating two days after plating , (c) three days after plating and (d) seven days after plating. Scale bars: 100 μm.

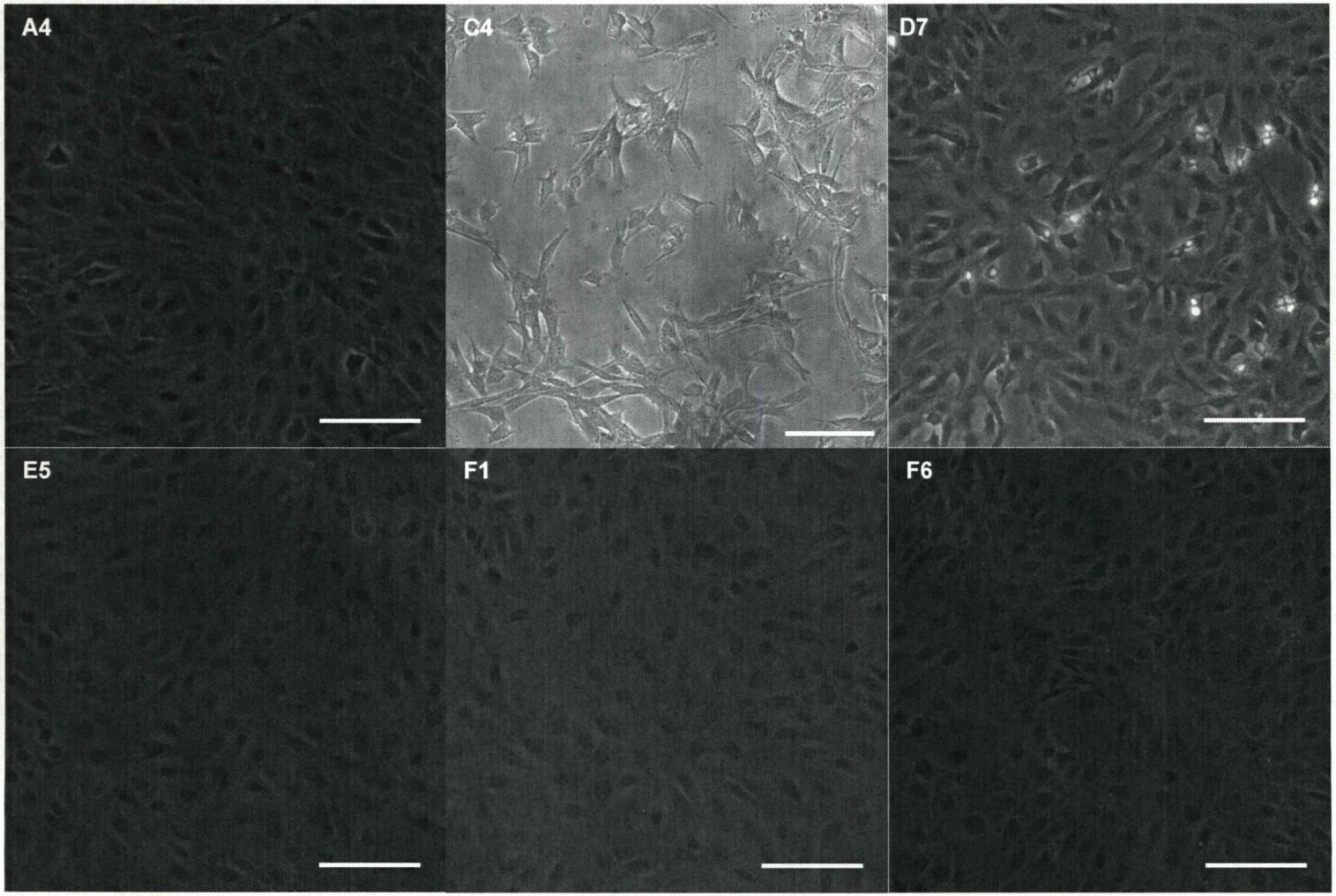


Figure 6-18(a) Phase contrast images of clones expanded from single cells. The clones shown are A4, C4, D7, E5, F1, and F6. Scale bar: 100 μm.

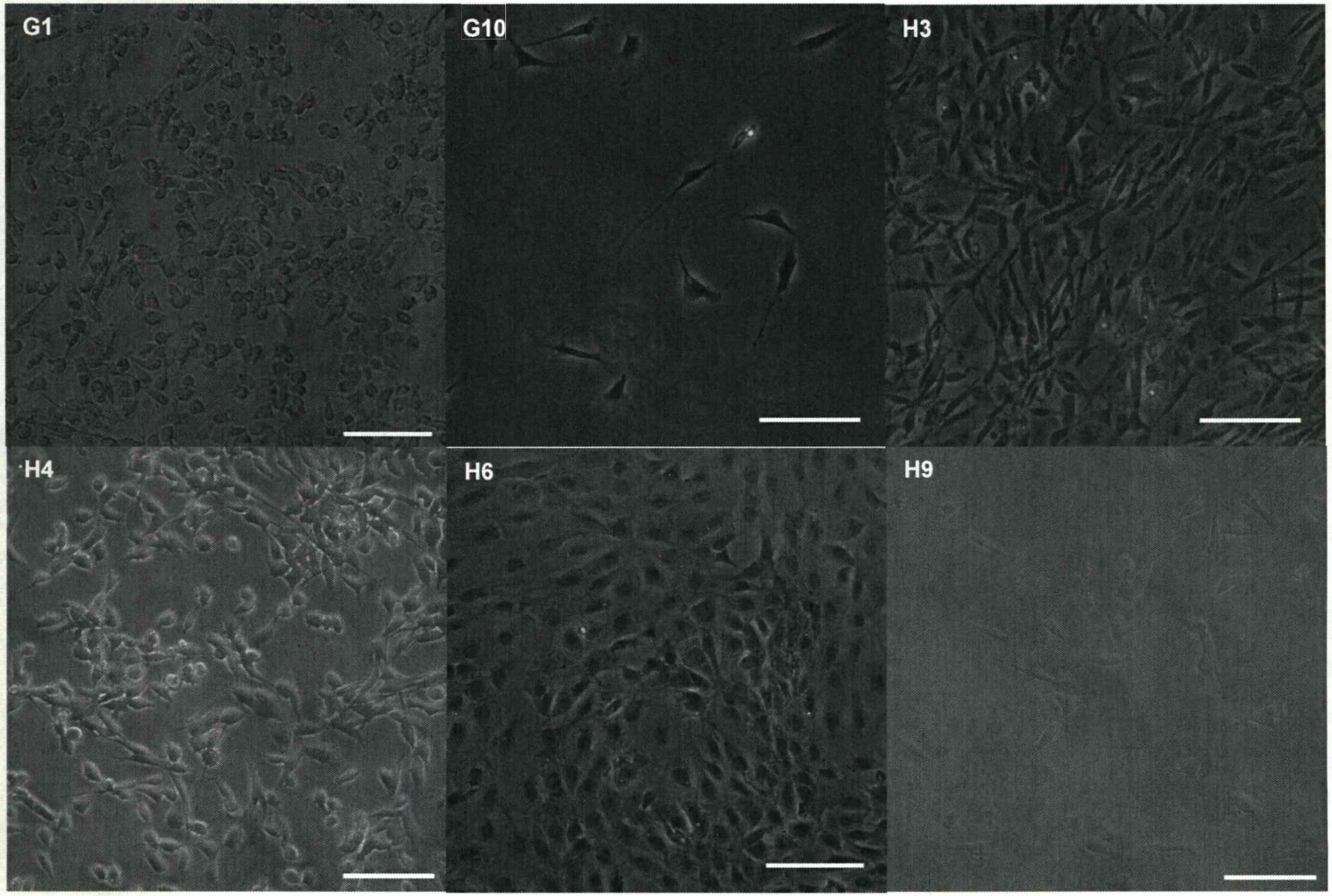


Figure 6-18 (b) Phase contrast images of clones expanded from single cells. The clones shown are G1, G10, H3, H4, H6, and H9. Scale bar: 100 μm.

a similar non differentiating morphology, however, six of the clones (C4, G10, H3, H9, G1, H4) presented a spindle shaped morphology. Note that some cells in these spindled cultures adopted a round morphology during division; this is common in most adherent cell types during mitosis (Gergely and Basto, 2008).

In one clone the morphology of the cells changed during expansion. This change was evident after approximately six weeks. The cells originally developed the spindle-shaped morphology and later presented more rounded morphology with indented nuclei (kidney-shaped) and a nuclear-cytoplasmic ratio of about 1:1, in which the nuclei occupied a large volume of the cells (Figure 6-19).

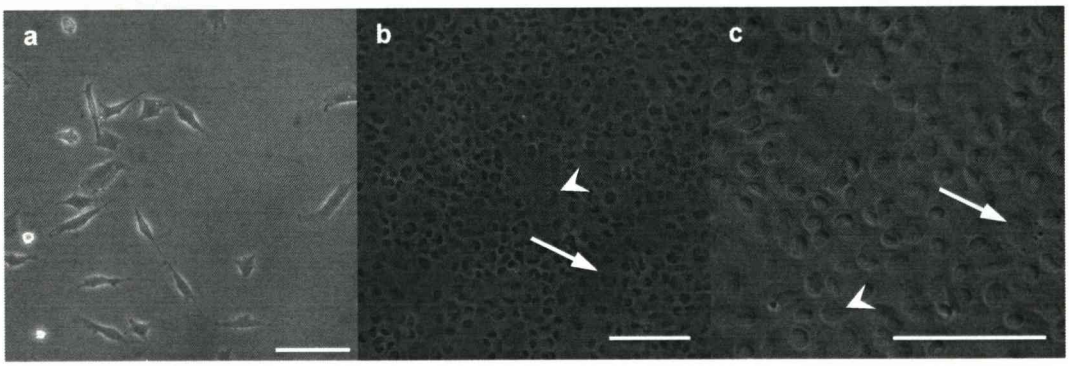


Figure 6-19 Phase contrast images of clone H9 after six weeks in culture. (a) Cells from clone H9 after two weeks in culture presented a spindle shaped morphology. (b-c) After six weeks in culture cells from H9 had acquired a rounded morphology with large nuclei (arrows) and some indented nuclei (arrowheads). Scale bars: 100 µm.

For further characterization the clones were analysed by immunostaining and RT-PCR. Six of the clones were analysed by RT-PCR for Pax-2, ksp-cadherin, THP, vimentin, desmin, cytokeratin, and megalin expression (Table 6-2). When the latter four markers were analysed the cDNA from one clone, H9, was found to have degenerated. One of the clones, A4, was positive for megalin, desmin, and vimentin but did not express cytokeratin. Another clone, G1, was positive for

Table 6-2 Summary of the results of the RT-PCR for the clones.

Marker	E5	H3	H9	A4	G1	D7
Pax-2	×	×	×	×	×	×
Ksp-Cad	×	×	×	×	×	×
THP	×	×	×	×	×	×
Vimentin	×	×	NA	✓	✓	×
Desmin	×	×	NA	✓	×	×
Cytokeratin	✓	✓	NA	×	×	×
Megalin	×	×	NA	✓	×	×

vimentin, but negative for cytokeratin, megalin and desmin. Two clones, E5 and H3, were positive for cytokeratin (Figure 6-20).

Eight of the clones derived from single cells were stained for αSMA after one month in culture. Two of the clones, A4 and D7, were completely negative for αSMA expression. Three clones, F6, H3, and H6, presented a high expression of αSMA. Clones E5, F1, and H9 presented few cells positive for αSMA.

Two of the clones were further analysed. The clone H6, which presented a heterogeneous morphology and different cell types, seemed to be present in the culture and E5 where the cells presented an homogenic morphology. The results showed that one of the clones (H6) presented many positive cells for αSMA. When this clone was stained for AP, enzyme activity was detected in some clusters of cells. Synaptopodin staining for this clone showed many positive cells for the marker, with higher expression in some binucleated podocyte-like cells (Figure 6-21a).

The other clone analysed for these markers was E5. Some cells in this clone were positive for αSMA. AP and synaptopodin were not expressed in any cell in this clone (Figure 6-21b).

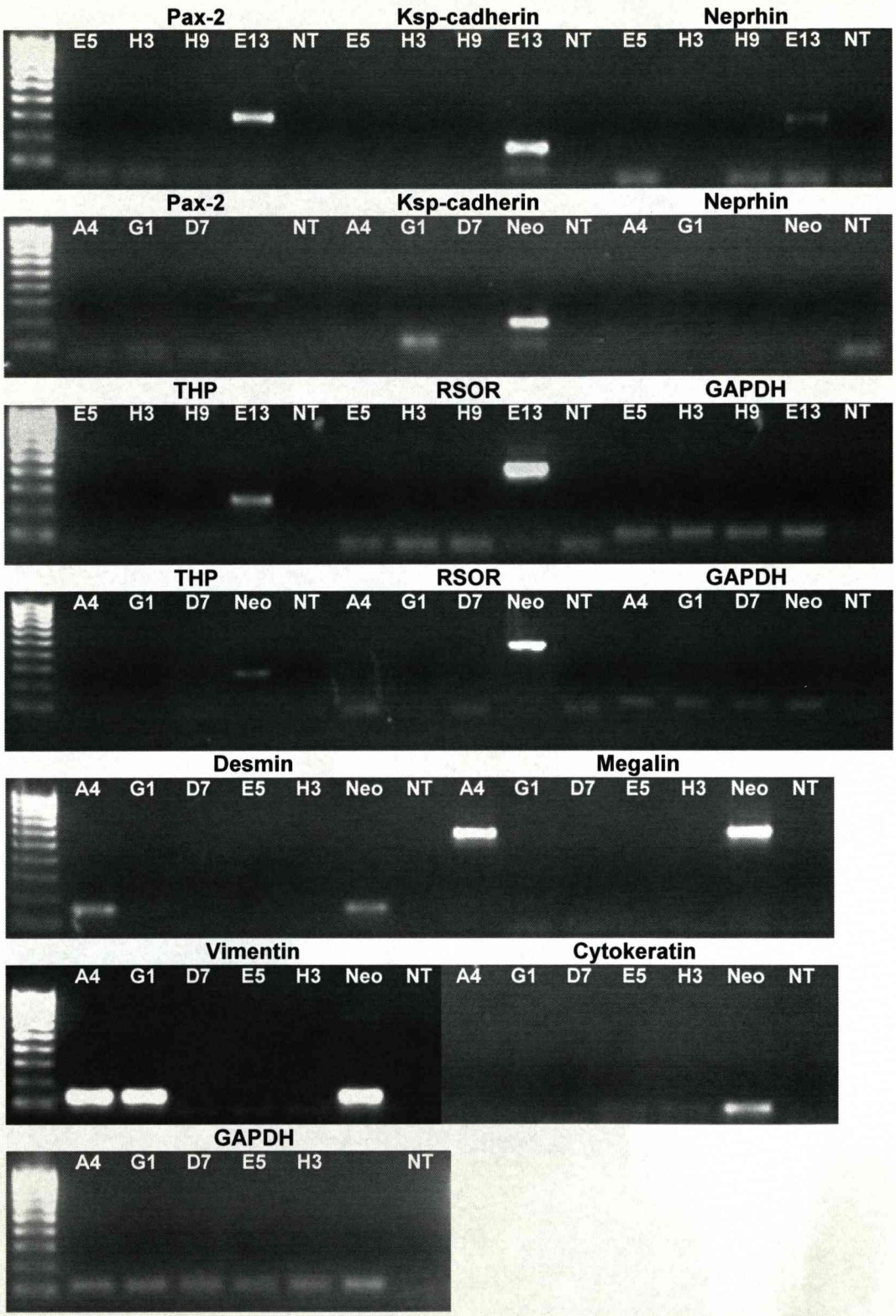


Figure 6-20 RT-PCR of cells expanded from clones from pKSC. The results of the RT-PCRs shown for the clones are summarised in Table 2. Kidneys from one week old neonate mice were used as positive controls, and reactions run with no template (NT) as negative controls.

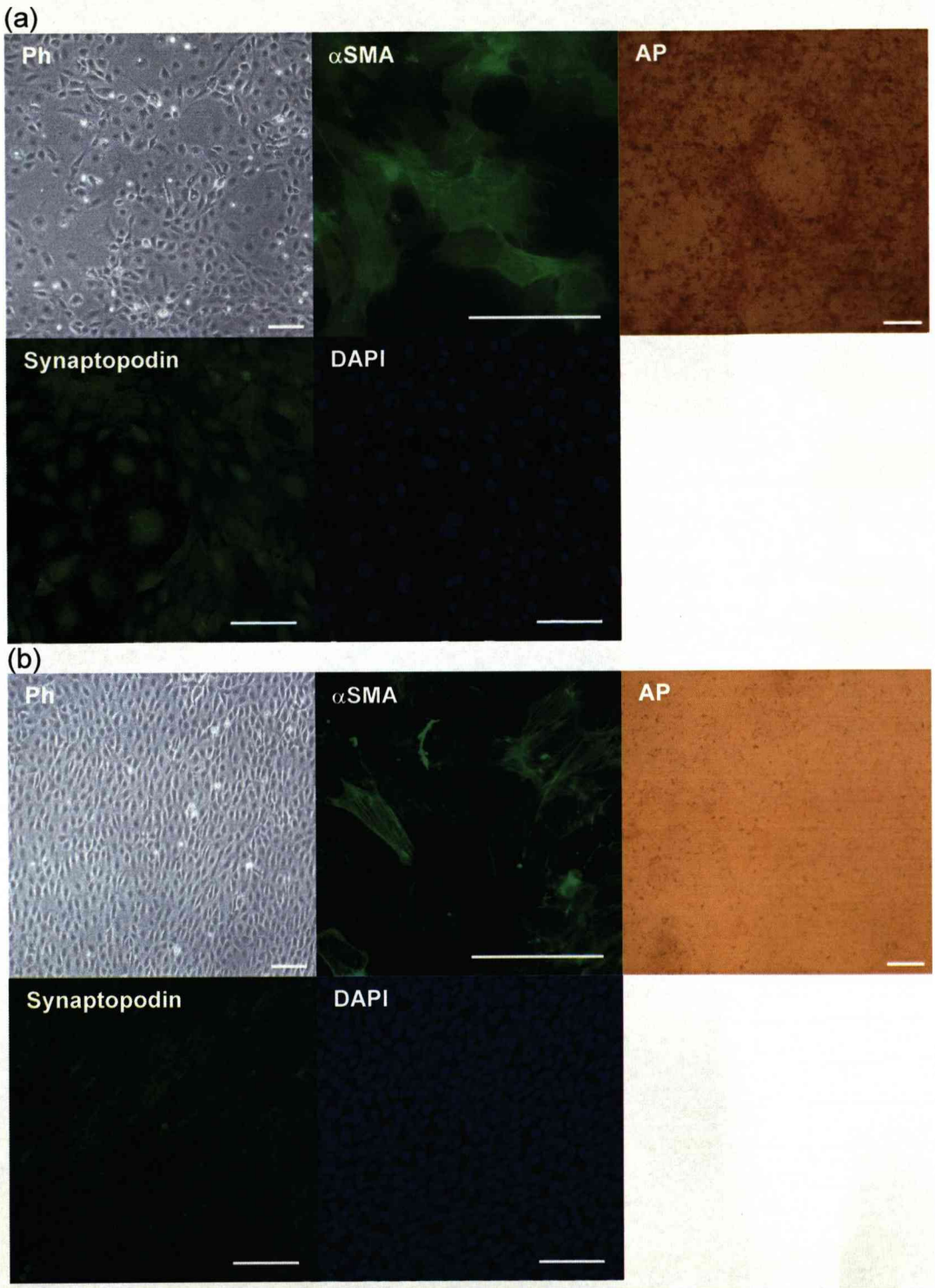


Figure 6-21 Micographs of clones E5 and H6 showing expression of αSMA and synaptopodin, and alkaline phosphatase activity. (a) Clone H6 showed expression of αSMA and synaptopodin and was positive for AP activity. (b) Clone E5 was postitive for αSMA but negative for AP and synaptopodin. Scale bars: 100 μm.

Growth curves of KSC and dKSC

The growth rates of pKSC and dpKSC were studied and compared. The results showed that the pKSC proliferated slightly quicker than the dpKSC. Linear regressions determined that the population doubling time for pKSC was 1.3 days (r^2 = 0.997) and the population doubling time for dpKSC was 1.5 days (r^2 = 0.990) (Figure 6-22).

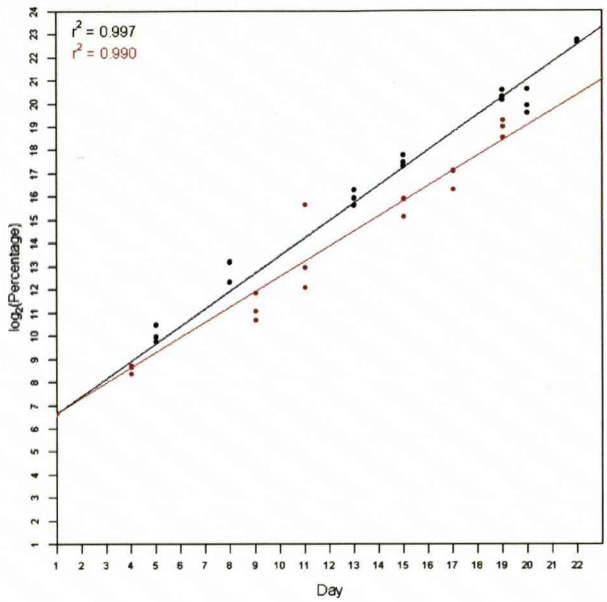


Figure 6-22 Graphs showing growth rates for pKSC and dpKSC. When cells cultures were analysed over a period of 21 days the pKSC (black), on fibronectin, were shown to proliferate at higher rate than dpKSC (red), on plastic. Doubling time for pKSC was 1.3 days (r^2 = 0.997) and for dpKSC was 1.5 days (r^2 = 0.990).

6.3 Discussion

In previous chapters, it was shown that a Pax-2 positive cell population could be isolated and expanded *in vitro* from neonatal mouse kidney. Based on their expression of the metanephric mesenchyme markers Pax-2, WT1, Sall1, and GDNF it was hypothesised that these cells might represent a population of kidney

stem cells. In order to determine whether this was the case, the work presented in this chapter sought to demonstrate that the cells had properties characteristic of stem cells, such as the ability to differentiate to mature cells types with expression of specific cell markers (Sagrinati *et al.*, 2006), self-renewal and clonogenicity.

The results showed that when cultured under conditions to promote their differentiation, the putative stem cell population could generate cells with the morphology of cultured podocoytes, and expressed some mRNAs and proteins characteristic of podocytes, mesangial cells and proximal tubule cells. Following culture under appropriate conditions, the population also showed the ability to generate non-renal cell types, as shown by the fact that the putative stem cells could give rise to adipocytes. To test their nephrogenic potential, the cells were combined with mouse kidney rudiment *ex vivo*, where it was found that the cells were able to condense around the UB, showing they were able to integrate into developing nephron-like structures. Finally, by generating single cell clones, at least one of which was able to give rise to multiple differentiated cell types, it was confirmed that the population of Pax-2 positive cells isolated from the neonatal mouse kidney contained true stem cells.

The differentiation potential of the putative kidney stem cell population

The differentiated pKSC (dpKSC) presented morphologies of more specialized kidney cells, including epithelial, mesenchymal, mesangial and podocyte-like morphologies. They also lost expression of Pax-2 and Sall1, but maintained WT1

and GDNF expression, and expressed a number of renal cell-specific markers, suggesting that the cells were differentiating to more specific renal cell types.

Two possible factors may have been influencing the cells in such a way as to cause them to differentiate: the medium and the substrate. The medium changed in two ways. First, the basal medium on which the pKSC were maintained was conditioned medium from STO feeders while the dpKSC were grown on high-glucose DMEM. It is well-established that embryonic stem cells can be maintained in an undifferentiated state when grown on a layer of fibroblast feeders (Evans and Kaufman, 1981a; Evans and Kaufman, 1981b). It is possible that the DMEM lacked certain factors released by the feeders that may maintain the cells in a state of self-renewal. Second, the DMEM contained a higher concentration of foetal calf serum, which is known to promote differentiation (Kruse and Tseng, 1993). Many differentiation protocols call for the use of high serum concentrations unless adequate cytokines are added (Lanza *et al.*, 2006), it may therefore be that the serum contains cytokines that promote differentiation.

It has been proposed that interaction of the cells with the substrate, and consequently cell shape, influences cell proliferation and cell differentiation (Huang and Ingber, 2000). When the pKSC were maintained, they were plated on fibronectin coated dishes, but in the "differentiating conditions" the cells were plated on top of the plastic of the dish with no coating. Fibronectin is a component of the kidney ECM (Ye *et al.*, 2004). It is possible, therefore, that fibronectin provides the Pax-2 positive cells from the papilla with an appropriate niche to support their self-renewal, which could explain why the cells lost

expression of Pax-2 and underwent spontaneous differentiation in the absence of fibronectin.

One of most striking differences in phenotype observed in the dpKSC was the appearance of cells with podocyte-like morphologies, including characteristics such as large cytoplasmic volumes, interdigitating processes, and binucleation, consistent with the criteria given by Shankland *et al.* (2007). Some of these cells expressed the protein synaptopodin , and also expressed higher levels of WT1 than their neighbours, which is a characteristic of podocytes (Dressler and Douglass, 1992).

Standard conditions for culturing podocytes *in vitro* result in dedifferentiation as indicated by the loss of processes and synaptopodin expression (Saleem *et al.*, 2002). These undifferentiated podocytes can be differentiated *in vitro* under certain conditions, but this results in a rapid growth arrest, preventing further expansion of the cells (Shankland *et al.*, 2007). It is therefore necessary to find alternatives, such as the use of immortalized podocyte lines (Mundel *et al.*, 1997b). The results in this chapter showed that differentiation of pKSC gave rise to cells with podocyte-like morphology and podocyte marker expression; hence, the use of pKSC and the differentiating conditions described could be an alternative avenue for generating podocyte cultures, which could be useful for studying podocyte biology and for toxicological studies.

The presence of mesangial cells in the dpKSC was also suggested by the mesangial-like morphology of some cells in the culture. This was reinforced by the expression of αSMA, which was absent prior to differentiation, and desmin.

Differentiation to tubular epithelial cells was also indicated by the presence of cells with an epithelial like morphology and the expression of cytokeratin 8, alkaline phosphatase, and megalin. However, the proximal tubule marker AQP1 was not expressed by the dpKSC. This might reflect the level of maturation of the cells, it is possible that these cells are in the process of differentiating to tubular cells, and that AP and megalin expression appears earlier than AQP1. Alternatively, it is possible that the *in vitro* culture conditions did not provide the necessary cues to induce AQP1 expression. AQP1 expression is essential for proximal tubular cell function and it will therefore be important in the future to determine if the cells can express AQP1 following integration into the kidney rudiments.

The absence of THP expression suggested that cells from the distal portion of the nephron were not present among the dpKSC. It has been shown that distal tubular cells in culture transdifferentiate to a more proximal phenotype thereby losing expression of THP and starting to express proximal marker proteins (Baer *et al.*, 1999). It is therefore possible that any distal tubular cells in dpKSC dedifferentiated to proximal tubular cells losing THP expression. Alternatively it could be that the differentiation conditions used did not promote differentiation to distal tubular cells.

Collecting duct cells were also apparently absent from dpKSC, as shown by the lack of AQP2 and Ksp-cadherin expression. The embryonic origin of the collecting duct is distinct from the rest of the kidney as it originates from ureteric bud rather than the metanephric mesenchyme (Vize *et al.*, 2003). The fact that

dpKSC could only generate MM derivatives, and not UB derivatives, supports the lineage tracing experiments in chapter one, that suggested the cells were derived from MM.

It was apparent that endothelial cells were not present on dpKSC due to the absence of the endothelial markers vWF and PECAM. It may be that the differentiating conditions used did not support endothelial differentiation, or it may be that pKSC do not have the potential to generate endothelium. The origin of renal endothelial cells is not clear. Some evidence suggests that the kidney endothelium originates extrarenally (Oliver *et al.*, 2002; Sariola *et al.*, 1984). While cultures of embryonic kidneys *ex vivo* do not form capillaries, it has been shown that mouse embryonic kidneys grafted onto quail chorioallantoic membrane develop glomerular capillaries that originate from the host membrane, suggesting that renal endothelial cells may have an extrarenal origin (Oliver *et al.*, 2002; Sariola *et al.*, 1984). However, Robert *et al.* (1996) demonstrated the development of glomerular capillaries of renal origin in embryonic mouse kidneys that had been grown *in vitro* and then grafted into the eyes of mice, which suggested that embryonic kidney may contain endothelial precursors.

Conditioned medium does not seem to be responsible for the pKSC phenotype as Pax-2 and Sall1 were not re-expressed by the cells when they were moved from the differentiating conditions back to fibronectin with STO-conditioned medium.

The pKSC also expressed many transcript markers of mature renal cells, despite displaying an homogeneous morphology lacking the characteristics of differentiated cell types. Although the transcripts are often reported to be markers

of mature renal cell types, many have been found to be expressed during the early stages of kidney organogenesis, at a time when mature cell types have not yet differentiated. For instance, megalin, which is reported to be a marker of proximal tubule cells, is known to be strongly expressed in the metanephric mesenchyme of E14 embryos (Assemat *et al.*, 2005).

As well as renal cells pKSC were also able to differentiate to adipocytes. This finding suggests that the pKSC might share some of the properties of mesenchymal stem cells, which can differentiate into multiple mesodermal cell types, including bone, fat, cartilage and muscle (Peister *et al.*, 2004). Both mesenchymal stem cells and metanephric mesenchyme are mesodermal derivatives, and under the appropriate culture conditions, might be expected to display a similar developmental potential. For instance, it has been shown that if human mesenchymal stem cells are transplanted to the site of the presumptive rat kidney before the metanephric mesenchyme has developed, they are readily incorporated into the developing kidney and are able to generate renal-specific cell types (Yokoo *et al.*, 2006).

The differentiation potential of single cell clones of pKSC

The pKSC were demonstrated to be highly clonogenic. Most of the clones, according to the analysed markers, showed little evidence of differentiation. Clone E5, which presented a homogeneous morphology under the microscope and expressed αSMA, appeared to have generated only one cell type, mesangial cells. This suggests that the cell from which the clone originated was unipotent.

Clone H6, in contrast, appeared to be multipotent. Cells expanded from this clone were able to differentiate to cells from different portions of the nephron, such as proximal tubules, mesangial cells and podocytes, as was shown by the expression of alkaline phosphatase, αSMA and synaptopodin respectively. Furthermore, cells from H6 presented a heterogenic morphology and different cell types were observed such as podocyte-like cells, mesangial-like cells and epithelial cells. The differentiation pattern of the clone H6 confirms that some pKSC were multipotent and could give rise to different cell types of the nephron.

Interestingly, one of the differentiated clones, H9, presented many cells that acquired indented or reniform nuclei. This nuclear morphology is not characteristic of any renal cell type, however, it has been observed in other cell types, such as cells from the islets of Langerhans (Hidaka *et al.*, 1979) or monocytes (Marshall *et al.*, 2006). To positively identify these cells a deeper analysis of specific markers would be required.

The nephrogenic potential of pKSC

During nephron formation in early kidney development the metanephric mesenchyme condenses around the ureteric bud (Ryan *et al.*, 1995). When labelled pKSC were mixed with disaggregated embryonic kidneys, they were similarly seen to condense around ureteric bud structures suggesting some nephrogenic potential. However, they did not seem to integrate into ureteric bud structures or developing nephrons, although it is possible that any pKSC that had integrated and begun to proliferate had lost the labelling dye and so could not be detected.

In summary, it has been shown that the population of potential KSC can be induced to differentiate to more specialized cell types, such as tubular epithelial cells or mesangial cells, by changing the culture conditions. These cells also have high clonogenic potential. These expanded populations also showed some potential to integrate in new developing kidney, but this aspect would need to be further studied to reach any formal conclusion. These renal cells can also be differentiated to extrarenal cell types such as adipocytes. In view of these properties, it appears that this population of cells isolated from neonatal kidney could be a stem cell population resident in the kidney, which can be isolated and manipulated *in vitro*.

Chapter 7: Final Discussion

In this study putative stem cells were isolated from postnatal mouse kidney, expanded *in vitro,* and their potential to generate specific renal cell types was evaluated. During this project mice were used as an animal model. In some species, such as humans, nephrogenesis is completed before birth, but in mice the kidney continues to undergo nephrogenesis after birth (Pierre *et al.*, 2008), and this makes it a good, accessible model. Whole kidneys were removed from the animal and studied, which provided a more comprehensive understanding of the different kidney cells populations than would have been possible analysing only samples from biopsies.

Adult stem cells exist in postnatal tissues and are involved in normal cell turnover and in regeneration after damage (Gupta and Rosenberg, 2008; Lanza *et al.*, 2006). However, in some organs, such as kidney or pancreas with a limited capacity of regeneration, the existence of adult stem cells remains controversial (Stanger *et al.*, 2007). The lack of a unique kidney stem cell marker has led researchers to find alternatives to identify and isolate potential stem cells from adult kidneys. For instance, some groups have identified stem cells based on the theory that stem cells in tissues are slow cycling and therefore retain BrdU (Maeshima *et al.*, 2006; Oliver *et al.*, 2004). Other groups have attempted to isolate stem cells from kidney using markers expressed in hæmatopoietic and other tissue stem cells such as CD133 (Bussolati *et al.*, 2005; Sagrinati *et al.*, 2006) or Sca-1 (Dekel *et al.*, 2006). A population of cells exhibiting stem cell

properties has been isolated with MACS® from human kidneys using the marker CD133 (Bussolati *et al.*, 2005; Sagrinati *et al.*, 2006). These cells also expressed the transcription factor Pax-2 (Bussolati *et al.*, 2005). During the first part of this study, the existence of a population of cells expressing the transcription factor Pax-2 in postnatal mouse kidney was investigated.

A population of Pax-2 positive cells, mainly located in tubules, was found in postnatal mouse renal papilla after nephrogenesis was completed. This fitted with the theory proposed by Oliver *et al.* (2004), that the renal papilla is a niche for kidney stem cells in rodents. This papillary Pax-2 population was a potential candidate for kidney stem cells with regenerative properties.

The renal papilla provides a unique environment due to the low pO_2 in this area (Oliver *et al.*, 2004). It has been shown that low oxygen tension controls adult organ-specific stem cell function (Cipolleschi *et al.*, 1993; Oliver *et al.*, 2004). An hypoxic environment can promote survival, proliferation, and the differentiation capacity of stem cells such as bone marrow and neuronal stem cells (Danet *et al.*, 2003; Morrison *et al.*, 2000). The renal papilla seems to present the ideal conditions to form a stem cell niche.

Pax-2 positive cells present in papilla seem to decrease when the mouse reach adulthood, however some Pax-2 cells seem to be expressed in renal papilla in adult mice and the evidence of Pax-2 present in adult mouse renal papilla has been shown before (Cai *et al.*, 2005). It would be important to test if Pax-2 positive cells present in adult mice have the same potential that Pax-2 isolated from kidneys of younger mice.

It is well established that the metanephric mesenchyme is committed to differentiate into nephrons whereas the ureteric bud is restricted to giving rise to the renal collecting system (Saxen, 1987; Vize *et al.*, 2003). It was therefore important to determine the origin of Pax-2 positive cells that remained in papilla after nephrogenesis. The hypothesis was that if papillary Pax-2 positive cells were derived from MM this would mean that they could have nephrogenic potential, whereas if they were derived from UB, they would not.

WT1-Cre/Rosa26R transgenic mice were used to determine the origin of Pax-2 papillary cells, because in these mice, cells expressing WT1 at any point during development would be permanently labeled with LacZ expression. It was found that many of the Pax-2 cells in the papilla of these mice also expressed β-galactosidase, and hence had expressed WT1 during development. This suggested that these cells had a MM origin and were therefore likely to be capable of forming nephrons.

The next step in the project was to isolate the Pax-2 positive cells from neonatal mouse kidney to later study their potential. MACS® is the preferred technique to isolate rare populations from tissues (Buageaw *et al.*, 2005; Pappas and Wang, 2007), and it has been successfully used to isolate several types of stem cells, including human kidney stem cells (Bussolati *et al.*, 2005; Collins *et al.*, 2001; De Coppi *et al.*, 2007; Qin *et al.*, 2004; Sagrinati *et al.*, 2006). Pax-2 was used to identify putative stem cells in murine kidneys, however, as the transcription factor is expressed in the nuclei of the cells it was necessary to first identify a surface marker to enable isolation of the Pax-2 positive cells using MACS®.

Lectins are carbohydrate specific binding proteins, and the fact that most cells contain carbohydrates on their surface make them suitable for cell isolation using MACS®. Lectins have been shown to show specific expression patterns on specific cell types in the kidney (Hanai *et al.*, 1994; Holthofer, 1983; Laitinen *et al.*, 1987; Schulte and Spicer, 1983). Among the twenty-one lectins tested, PNA showed specific binding to most of the Pax-2 cells present in kidney during development. It was therefore chosen to enrich the population of Pax-2 cells from the whole kidney population.

PNA MACS® separation successfully enriched Pax-2 cells, however, it was also found to be possible to obtain high numbers of Pax-2 positive cells simply by plating disaggregated cells from kidney and then culturing the cells for two weeks. If the Pax-2 expressing cells were indeed a stem cell population, then they could have had a proliferation advantage over differentiated cells. This could explain why, after two weeks in culture, the numbers of Pax-2 expressing cells were very similar in cells selected with PNA and in disaggregated kidney cells on which no isolation procedure had been performed. MACS® is an expensive and time consuming technique. Overall, it seemed that enrichment of Pax-2 cells using PNA coated magnetic beads was not worthwhile, at least for use in early neonates, in which there are high numbers of cells expressing Pax-2. However, it is possible that the technique could be more successful in older mice, in which Pax-2 numbers are decreased. It may also be possible to improve the result of MACS® enrichment of the Pax-2 cells by performing a negative selection. The use of a lectin that only binds Pax-2 negative cells would remove these cells, and

thus further enrich the Pax-2 positive cells. A possible candidate indicated by the results in Chapter 4 would be PHA-L.

The next step was then to explore the possibility that Pax-2 cells present in neonatal kidneys may be a population of stem cells. Stem cells can be maintained self-renewing in culture, giving rise to daughter cells that are also stem cells, or they can be induced to differentiate to specific cell types. In order to study stem cells *in vitro*, the culture conditions required to maintain them undifferentiated needed to be optimized.

Conditioned medium from STO fibroblast containing 2.5% serum proved to be a better medium in which to grow the cells than Advanced DMEM. STO fibroblasts are widely used to grow embryonic stem cells and it has been shown that STO fibroblasts release growth factors into the medium that may be essential to maintain stem cells in an undifferentiated state (Ouyang *et al.*, 2007; Talbot *et al.*, 1994; Xu *et al.*, 2001). Factors contained in the STO-conditioned medium could be responsible for cell proliferation. However, the conditioned medium also contained 2.5% fetal calf serum, and it may be that the serum contained chemical signals, such as growth factors, that were responsible for promoting cell survival and proliferation. It is very likely that the combination of both factors was responsible for the improvement in cell expansion.

Adhesion to the ECM is essential for cell survival and proliferation (Xu *et al.*, 2001). The adhesion of putative stem cells to different substrates was studied in order to test which was the most suitable for promoting proliferation and cell

survival *in vitro*. It was found that fibronectin supported long term proliferation and expansion of cells maintaining expression of MM markers.

The fact that under optimized conditions the cells were able to proliferate and maintain expression of MM markers supports the hypothesis that cells expanded from disaggregated kidneys are a potential stem cell population. This technique may be used as an alternative to select stem cells instead of the MACS® isolation. Gupta *et al.* (2006) previously reported a similar approach of isolating stem cells from kidney using optimized conditions that promoted their proliferation.

One of the properties of stem cells is that they can differentiate, acquiring morphological, phenotypical, and functional features of specific cell types (Lanza *et al.*, 2006). The differentiation potential of the putative kidney stem cells (pKSC) isolated in this study needed to be assessed in order to determine whether they were truly stem cells.

It was hypothesised that, given the renal origin of pKSC, they would already have some renal commitment, and that it would be feasible to differentiate them to renal specific cell types. To promote differentiation of pKSC the culture conditions of the cells were changed. The cells were plated onto plastic dishes with no coating and STO-CM with 2.5% FCS was then substituted for medium containing 10% FCS. Under these new conditions the cells underwent morphological changes and lost expression of the MM markers Pax-2 and Sall1. This change in the phenotype suggested that the cells could be differentiating to more specific In this study putative stem cells were isolated from postnatal mouse

kidney, expanded *in vitro,* and their potential to generate specific renal cell types was evaluated.

Adult stem cells exist in postnatal tissues and are involved in normal cell turnover and in regeneration after damage (Gupta and Rosenberg, 2008; Lanza *et al.*, 2006). However, in some organs, such as kidney or pancreas with a limited capacity of regeneration, the existence of adult stem cells remains controversial (Stanger *et al.*, 2007). The lack of a unique kidney stem cell marker has led researchers to find alternatives to identify and isolate potential stem cells from adult kidneys. During the first part of this study, a population of Pax-2 positive cells, mainly located in tubules, was found in postnatal mouse renal papilla after nephrogenesis was completed. This fitted with the theory proposed by Oliver *et al.* (2004), that the renal papilla is a niche for kidney stem cells in rodents. This papillary Pax-2 population was a potential candidate for kidney stem cells with regenerative properties.

It was found that many of the Pax-2 cells in the papilla of WT1-Cre transgenic mice also expressed β-galactosidase, and hence had expressed WT1 during development. This suggested that these cells had a MM origin and were therefore likely to be capable of forming nephrons.

PNA MACS® separation successfully enriched Pax-2 cells, however, it was also found to be possible to obtain high numbers of Pax-2 positive cells simply by plating disaggregated cells from kidney and then culturing the cells for two weeks. If the Pax-2 expressing cells were indeed a stem cell population, then they could have had a proliferation advantage over differentiated cells. This could

explain why, after two weeks in culture, the numbers of Pax-2 expressing cells were very similar in cells selected with PNA and in disaggregated kidney cells on which no isolation procedure had been performed. MACS® is an expensive and time consuming technique. Overall, it seemed that enrichment of Pax-2 cells using PNA coated magnetic beads was not worthwhile, at least for use in early neonates, in which there are high numbers of cells expressing Pax-2. However, it is possible that the technique could be more successful in older mice, in which Pax-2 numbers are decreased. It may also be possible to improve the result of MACS® enrichment of the Pax-2 cells by performing a negative selection. The use of a lectin that only binds Pax-2 negative cells would remove these cells, and thus further enrich the Pax-2 positive cells. A possible candidate indicated by the results in Chapter 4 would be PHA-L.

It was found that fibronectin and STO-conditioned medium supported long term proliferation and expansion of cells maintaining expression of MM markers. The fact that under optimized conditions the cells were able to proliferate and maintain expression of MM markers supports the hypothesis that cells expanded from disaggregated kidneys are a potential stem cell population. This technique may be used as an alternative to select stem cells instead of the MACS® isolation. Gupta *et al.* (2006) previously reported a similar approach of isolating stem cells from kidney using optimized conditions that promoted their proliferation.

Standard conditions for culturing podocytes *in vitro* result in dedifferentiation as indicated by the loss of processes and synaptopodin expression (Saleem *et al.*, 2002). These undifferentiated podocytes can be differentiated *in vitro* under

certain conditions, but this results in a rapid growth arrest challenging expansion of the cells (Shankland *et al.*, 2007). It is therefore necessary to find alternatives, such as the use of immortalized podocyte lines (Mundel *et al.*, 1997b). The results in this study showed that differentiation of pKSC gave rise to cells with podocyte-like morphology and podocyte marker expression, hence the use of pKSC and the differentiating conditions described could be an alternative avenue for generating podocyte cultures.

pKSC could also be differentiated into other renal cell types such as mesangial and proximal tubular cells. The pKSC seemed to lack the capacity to form distal tubular cells, collecting duct cells or endothelial cells. It may be that the conditions used to differentiate the cells do not promote differentiation to these cell types or it could be that pKSC do not have the ability to form them.

The pKSC also showed the capacity to form extrarenal cell types such as adipocytes. One characteristic of mesenchymal stem cells is that they have the potential to generate fat, cartilage, bone and muscle (Peister *et al.*, 2004). Sagrinati *et al.* (2006) isolated a population of cells from human kidney using CD133 to select them. These cells with stem cells characteristics were able to generate renal tubular cells, osteocytes, adipocytes, and neural cells. It may be that pKSC from mice are an equivalent population to those isolated by Sagrinati *et al.* (2006) in human kidneys.

It is possible that pKSC may have some nephrogenic potential *ex vivo* as shown by their condensation around UB structures, resembling early kidney development events. However, they did not appear to integrate into UB structures

or nephrons. It may be that the cells do not have the potential to integrate, or it may be that following integration and proliferation the cells lost the tracking label, meaning that they could no longer be visualized. If this was the case, GFP expressing cells may be a solution to overcome the loss of the cell tracker after proliferation

The cells were also highly clonogenic and some of the clones generated from single cells were able to give rise to at least three different cell types. This proved that the pKSC population contained multipotent cells, which were able to generate different cell types, rather than simply being a mix of different progenitors with limited differentiation potentials.

Some future work will need to be done in order to assess the potential of these pKSC in regenerative medicine. In this study has been shown that stem cells with the potential to generate various renal-specific cell types are present in the postnatal mouse kidney. However, although these renal-specific cells have a similar morphology and expression profile to those present in the kidney neprhons, it will be important to confirm that the stem cell-derived podocytes, mesangial cells and proximal tubule cells also have the ability to function in the same way as their *in vivo* counterparts.The ability of the cells to integrate into developing kidney and form kidney structures will also need to be further studied. It seems very unlikely that if these cells cannot integrate into embryonic kidney when nephrogenesis is still occurring, that they would be able to do so in adult kidneys.

Furthemore, to assess if these pKSC are likely to have a future use in the treatment of human kidney disease, it will be important to investigate if these cells have any potential to regenerate structures or improve the renal function in animal models, as has been previously demonstrated with other stem cells (Bussolati *et al.*, 2005; Challen *et al.*, 2006; Dekel *et al.*, 2006; Gupta *et al.*, 2006; Kitamura *et al.*, 2005; Morigi *et al.*, 2008).

An issue with the current study is that the Pax-2 positive cells were isolated from neonatal mouse kidneys that were still undergoing nephrogenesis, and therefore contained two populations of Pax-2 positive cells: a population in the cortex that plays in role in the formation of new nephrons, and a population in the papilla. It is therefore possible that the Pax-2 positive cells isolated here were from the cortex. However, if the cells were from the cortex, they would have limited use in any future stem cell-based therapy because they are only present in the kidney during its development. It would thus be important in the future to investigate if a similar Pax-2 positive population to the one characterised here could be isolated from kidneys that had completed nephrogenesis, because at this stage, the only Pax-2 positive cells present are in the papilla.

In conclusion, the work presented in this thesis has demonstrated that neonatal mouse kidneys contain a population of Pax-2 expressing cells that display characteristics of stem cells such as the ability to self-renew long term in culture, the ability to differentiate, and clonogenicity, suggesting that this population is very likely to be kidney stem cells similar to those previously isolated in humans (Bussolati *et al.*, 2005; Sagrinati *et al.*, 2006). These cells were able to give rise to

specific renal cell types that would be required to generate a functioning nephron. Furthermore, the characteristic expression of Pax-2 by these cells was also observed in the kidneys of 2–3 week old and, to a lesser extent, adult mice suggesting that this population may be resident in mature or even adult kidney.

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